Molecular regulation of neuroblast migration in the postnatal brain and intracellular trafficking of Diacylglycerol lipase

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A thesis for the degree of Doctor of Philosophy

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Abstract

During development and after birth neural stem cells in the subventricular zone (SVZ) generate neuroblasts that migrate along the rostral migratory stream (RMS) to populate the olfactory bulb (OB) with neurons. Multiple factors promote neuroblast migration, but the contribution that many of these make to guidance within the intact RMS is not known. In the first part of this present study we have characterised in detail how endocannabinoid (eCB), fibroblast growth factor receptor (FGFR) and brain-derived neurotrophic factor (BDNF) signalling regulate motility and guidance, and also determined whether any of these receptors operate in a regionally restricted manner. We used *in vivo* electroporation in postnatal mice to fluorescently label neuroblasts, followed by live cell imaging to detail their migratory properties. Cannabinoid receptor antagonists rendered neuroblasts less mobile, and when they did move guidance was lost. Similar results were obtained when eCB synthesis was blocked with diacylglycerol lipase (DAGL, enzymes that are responsible for the synthesis of the eCB receptor ligand 2-AG) inhibitor. Importantly eCB function is required for directed migration at both ends of the RMS. Likewise, inhibition of BDNF signalling disrupted motility and guidance in a similar manner along the entire RMS. In contrast, altering FGFR signalling inhibits motility and perturbs guidance, but only at the beginning of the stream. Inhibition of FGFR signalling *in vivo* also reduces the length of the leading process on migratory neuroblasts in a graded manner along the RMS.

In the second part of this study we focus on the intracellular trafficking of DAGLs. Apart from their function in neuroblast migration as shown above, DAGLs are also involved in axonal growth, guidance and synaptic signalling. We developed a new construct which allows the surface labelling of DAGLα. By expressing this construct in neurons, we found DAGLα co-localizes with Homer, a postsynaptic marker. An antibody feeding assay revealed that DAGLα undergoes constitutive endocytosis in cultured hippocampal neurons and COS-7 cells. Detailed studies showed that DAGLα co-localizes with early endosome markers and undergoes recycling in COS-7 cells. This constitutive endocytosis and recycling of the enzyme is likely to be responsible for the distinct localization pattern of DAGLα in neurons.

In summary, these results describe the important role of eCB, FGFR and BDNF signalling in neuroblast migration in the postnatal brain. Also it is the first time it has been shown that DAGLα undergoes constitutive endocytosis and recycling inside of the cell.

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Publications arising from this thesis

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Abbreviations

2-AG	2-arachidonoylglycerol
AA	Arachidonic acid
aa	Amino acid
ABHD6	α and β-hydrolase domain-containing protein-6
AD	Alzheimer's disease
AEA	N-arachidonoylethanolamide
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Anandamide	N-arachidonoylethanolamide
BDNF	Brain derived neurotrophic factor
BrdU	Bromodeoxy-Uridine
Ca^{2+}	Calcium
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CamKII	Calmodulin dependent protein kinase II
CB	Cannabinoid
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CC	Coiled-coil
cDNA	Complementary deoxyribonucleic acid
CGN	Cerebellar granule neuron
CNS	Central nervous system
CP	Choroid plexus
Cre	Cyclic Recombinase
CREB	cAMP response element binding protein
CSF	Cerebrospinal Fluid
CTxB	Cholera Toxin Subunit B
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DCX	Doublecortin
Dbox	Destruction box

DG	Dentate Gyrus
DHPG	(S)-3,5-dihydroxyphenylglycine
DIV	Days *in vitro*
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic Acid
DN	Dominant negative
dpe	Days post electroporation
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
E	Embryonic day
eCB	Endocannabinoid
ECM	Extracellular Matrix
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
eIPSCs	Evoked inhibitory postsynaptic currents
FAAH	Fatty acid amide hydrolase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FMRP	Fragile X mental retardation protein
GABA	γ-Aminobutyric acid
GC	Granule cell
GCL	Granule cell layer
GDF11	Growth and differentiation factor-11
GDNF	Glial cell line derived neurotrophic factor
GFAP	Glial fibrilliary astrocyte protein
GFP	Green fluorescent protein
GL	Granular layer
Glu	Glutamate
GPCR	G-Protein Coupled Receptor

GTP	Guanosine triphosphate
GTPases	(GTP)-binding proteins
HEK	Human embryonic kidney
HGF	Hepatocyte growth factor
IGF	Insulin growth factor
LPA	Lysophosphatidic acid
LPI	Lysophosphatidylinositol
LTD	Long term depression
LTP	Long term potentiation
IRES	Internal ribosome entry site
LV	Lateral ventricle
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-Activated Protein Kinase
mDia	Mammalian Diaphanous
mGluR	Metabotropic glutamate receptor
miRNA	micro-RNA
mRNA	Messenger RNA
N	Amino
NAE	N-acylethanolamine
NAPE	N-arachidonoyl phosphatidylethanolamine
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NS	Neural stem
OB	Olfactory bulb
P	Postnatal day
p75NTR	p75 neutrophin receptor
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PI	Phosphatidylinositol

PIP2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PP	Proline-rich
PSA	Polysialic Acid
PSA-NCAM	Polysialylated neural cell adhesion molecule
PSD-95	Postsynaptic density 95
RG	Radial glia
RMS	Rostral migratory stream
RNA	Ribonucleic Acid
Robo	Roundabout
S	Serine
Ser	Serine
SGZ	Subgranular zone
shRNA	Small hairpin ribonucleic acid
SVZ	Subventricular zone
TCF	T cell factor
THC	Δ9–tetrahydrocannabinol
THL	Tetrahydrolipstatin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VGCC	Voltage-gated calcium channel
VZ	Ventricular zone
WT	Wild type
YFP	Yellow fluorescent protein

Chapter 1 Introduction

‘The brain is a wonderful organ’ as written by American poet Robert Frost, ‘It starts working the moment we get up in the morning, and doesn’t stop until we get to the office’. From the scientific point of view, the human brain is wonderful not only because it enables the poet to write poetry and make jokes. It is wonderful because it is the most complex organ that we are trying to understand, using the brain itself. The complexity of the human brain can be presented by few facts: it is composed of approximately 86 billion neurons (Herculano-Houzel, 2009), with each of them has on average 7000 synaptic connections to other neurons. A typical adult human brain is estimated to have over 100 trillion synapses (Drachman, 2005). Apart from the numbers, it is amazing how these individual neurons and synapses connect to each other and make a functional brain. A functional human brain requires appropriate development that includes the development of the birth of neurons, their migration to appropriate locations, development of axons and formation of appropriate connections. The system then has to function and it does so largely via synaptic transmission. However, complex learning and memory are the features that set us apart from other animals and the basic features here include synaptic plasticity and cellular plasticity in the form of neurogenesis. In this thesis I will introduce the endocannabinoid (eCB) signalling and highlight the important contribution it plays in many aspects of the developing and functioning brain.

1.1 The eCB system; a historical perspective

1.1.1 Discovery of the eCB system

The medicinal use of *Cannabis sativa* was first discovered by Shen Nong, a legendary emperor and father of Chinese medicine who tasted about 365 herbs and died of a toxic overdose over 4000 years ago. The medicinal properties of cannabis were first recorded in ‘Shen Nong’s Herbal Classic’, a book attributed to him. While the exact dates of the life of Shen Nong and the completion of the book are not known, the earliest therapeutic applications of cannabis can be found in literature dated over 2700 years ago (Russo et al, 2008). However, the structure of Δ9–tetrahydrocannabinol (THC), the major psychoactive ingredient of cannabis was only solved 50 years ago (Mechoulam & Gaoni, 1965) and it triggered a cascade of crucial discoveries leading to the characterisation of the eCB signalling system.

The identification of the specific binding site of THC in the brain was followed by the cloning of the first CB receptor, cannabinoid receptor 1 (CB1) from a rat brain cDNA library (Matsuda et al, 1990). Another CB receptor, cannabinoid receptor 2 (CB2) which shares 48% sequence similarity with CB1 was cloned from a leukemic cell line shortly afterwards (Munro et al, 1993). The CB receptors are seven trans-membrane G-protein coupled receptors (GPCRs). The CB1 receptor is the most abundant GPCR in the CNS. High levels of expression of the CB1 receptor were found in the olfactory system, the hippocampal formation, the basal ganglia, the cerebellum and the neocortex in rat brain (Egertova & Elphick, 2000). It mainly localized in nerve fibre systems and axon terminals but not in neuronal somata (Egertova & Elphick, 2000). However, it can also be found in peripheral organs such as liver and skeletal muscle (Matias & Di Marzo, 2007). The CB2 receptor is instead mainly expressed in immune system cells such as B cells, monocytes, neutrophils and T cells (Howlett, 2002). However, lower levels are believed to be present in some restricted areas of the CNS (Duff et al, 2013; Howlett, 2002). A more recent study supports broader CNS roles for the CB2 receptor, with the CB2 receptor immunoreactivity detected in various neuronal and glial cell types in a number of brain regions in the adult rat brain, suggesting the possible roles of this receptor may play in the brain (Gong et al, 2006).

Identification of the CB receptors and further studies on their roles lead to the discoveries of their endogenous ligands (endocannabinoids; eCBs). 2-arachidonoylglycerol (2-AG), the most abundant eCB in the brain, was identified as an eCB in 1995 (Mechoulam et al, 1995; Sugiura et al, 1995). This was followed by the cloning of monoacylglycerol lipase (MAGL), the enzyme required for 2-AG degradation (Saario & Laitinen, 2007), and of two *sn*-1-selective diacylglycerol lipases (DAGLα and DAGLβ), the enzymes responsible for 2-AG synthesis (Bisogno et al, 2003). These discoveries greatly contributed to the development of new therapeutic strategies targeting components of the eCB system. Besides 2-AG, several other endogenous lipids have been proposed to function as eCBs. These include derivatives of arachidonic acid conjugated with ethanolamine or glycerol such as N-arachidonoylethanolamide (AEA), also known as anandamide which was the first eCB to be identified (Devane et al, 1992). Noladin ether (CB1 agonist), and virhodamine (CB1 antagonist/CB2 agonist) are also putative eCBs (Bisogno et al, 2005; Hanus et al, 2001). 2-AG and AEA, the two main eCBs found in the brain, are so far the best-characterised eCBs. By acting on the two $G_{i/o}$ protein-coupled CB receptors (CB1 and CB2), they trigger

downstream signalling cascades regulating crucial events in brain development like axonal growth and guidance, neurogenesis, and retrograde signalling at synapses (Oudin et al, 2011b).

In this thesis, the eCB system is used to collectively describe the CB receptors (CB1 and CB2), their two well-studied endogenous ligands (2-AG and AEA), and the enzymes involved in their synthesis and degradation (e.g. DAGLs and MAGL) which will be discussed in detail below.

1.1.2 Synthesis and degradation of the eCBs

2-AG is an ester containing an arachidonic acid (AA) chain at the *sn*-2 position of the glycerol backbone. It was originally studied as an intermediate product in the AA synthesis and in 1995, two studies revealed that 2-AG could bind to the CB receptors in canine intestines and rat brain, suggesting its important role as an eCB and raised interest of the field to further understand its synthesis and degradation (Mechoulam et al, 1995; Sugiura et al, 1995). Several studies reported the abundance of 2-AG in the CNS shortly afterwards (Bisogno et al, 1999b; Kondo et al, 1998; Stella et al, 1997). For example, Stella *et al.* reported that 2-AG is present in the rat brain in amounts 170 times higher than AEA, another main eCB in the brain (Stella et al, 1997). Bisogno *et al*. who measured 2-AG and AEA levels in 9 different brain regions also found 2-AG to be more abundant than AEA (Bisogno et al, 1999a).

Synthesis of 2-AG can be mediated by DAGL from AA-containing diacylglycerol (DAG) and the first evidence was found in human platelets in 1979 (Bell et al, 1979). Several other groups also attempted to characterize the DAGL in the context of AA synthesis pathway and found that the enzyme has an optimal pH of 7.0 and its action at the *sn*-1 position can lead to the release of AA (Chau & Tai, 1981; Okazaki et al, 1981; Prescott & Majerus, 1983; Sutherland & Amin, 1982). However, DAGL remained to be an elusive enzyme until in 2003 our lab reported the cloning of two *sn*-1-selective DAGLs (DAGLα and β) (Bisogno et al, 2003). Both enzymes are highly specific *sn*-1 DAGLs and can release 2-AG in response to calcium influx. They can be inhibited by RHC80267 and tetrahydrolipstatin (THL), drugs known as DAGL inhibitors (Bisogno et al, 2003). Interestingly, DAGLs are expressed in the axonal tracts during development, but the expression shifted to dendrites in the adults, suggesting the important development-dependent switch in the role of the DAGLs (Bisogno et al, 2003). Knock-out mice studies further support the role of the enzymes in regulating the steady state levels of 2-AG throughout the body, with DAGLα and DAGLβ making different contributions in different tissues (Gao et al, 2010). The role of DAGLα in retrograde eCB signalling in the CNS was also revealed by two independent knock-out mice

studies (Gao et al, 2010; Tanimura et al, 2010). Depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE), two related forms of short-term synaptic plasticity, were lost in hippocampus, cerebellum and striatum in DAGLα-/- but not in DAGLβ-/- mice (Gao et al, 2010; Tanimura et al, 2010). All these studies together support the important roles of DAGLs, especially DAGLα, as a component of the eCB signalling system in the CNS.

DAGLs have been identified as the major enzymes for 2-AG synthesis. Other putative pathways involved in 2-AG synthesis have also been reported, for example, 2-AG can also be produced by sequential hydrolyses of phosphatidylinositol (PI) via Lyso PI and 2-arachidonoyl lysophosphatidic acid (LPA) can also be converted to 2-AG via a phosphatase (Figure 1-1A). However, the relative importance of these pathways is less clear and they may depend on the stimuli and type of cells (Ueda et al, 2011).

MAGL is the principal enzyme for 2-AG degradation. MAGL is a serine hydrolase and it cleaves 2-AG into AA and glycerol and this pathway is considered to be responsible for ~85% of the 2-AG hydrolysing activity in the brain (Ueda et al, 2011). Inhibiting MAGL activity by MAGL inhibitors *in vivo* can lead to a significant increase of endogenous 2-AG (Blankman et al, 2007; Ueda et al, 2011). Apart from MAGL, α and β-hydrolase domain-containing protein-6 (ABHD6) and ABHD12 and fatty acid amide hydrolase (FAAH) can also hydrolyze 2-AG in the brain (Figure 1-1B). FAAH can hydrolyze both 2-AG and AEA, however, its role in 2-AG degradation is minor in the brain (Blankman et al, 2007; Ueda et al, 2011).

Anandamide or AEA is another well studied eCB which was first identified as an eCB in a screen for endogenous ligands for the CB receptor in 1992 (Devane et al, 1992). It inhibited the specific binding of a radiolabelled cannabinoid probe to synaptosomal membranes in a manner typical of competitive ligands, suggesting it may function as a natural ligand for the CB receptor (Devane et al, 1992). AEA belongs to the N-acylethanolamine (NAE) family, a group of bioactive lipids. Despite the similarity of structures of 2-AG and AEA, they are synthesized by distinct pathways. AEA can be synthesized from N-arachidonoyl phosphatidylethanolamine (NAPE) by multiple pathways, some of which still need to be better understood (Wang & Ueda, 2009). The degradation of AEA is mainly carried out by FAAH, which converts AEA into ethanolamine and AA. Inhibition of FAAH activity by pharmacological tools or genetically knock-out of FAAH both lead to

elevated AEA levels in the brain (Hwang et al, 2010). Like MAGL, which is the main enzyme responsible for 2-AG degradation, FAAH is also pursued as a therapeutic target for neuroprotection (Gaetani et al, 2009; Hwang et al, 2010). Inhibiting FAAH has been shown to be able to improve the learning and memory performances in *fmr1* knock-out mice, a model for Fragile X syndrome, indicating that the eCB system is a promising target for Fragile X syndrome treatment (Qin et al, 2015).

Overall, 2-AG and AEA are the two main eCBs in the brain. Due to the abundance of 2-AG over AEA in the brain and the fact that 2-AG acts as a full agonist of both the CB1 and CB2 receptors, it is argued that 2-AG is indeed the 'true' eCB (Sugiura et al, 1999). Better understanding of the synthesis and degradation of 2-AG and AEA will lead to development of new pharmacological tools targeting the eCB signalling (Gaetani et al, 2009; Hwang et al, 2010).

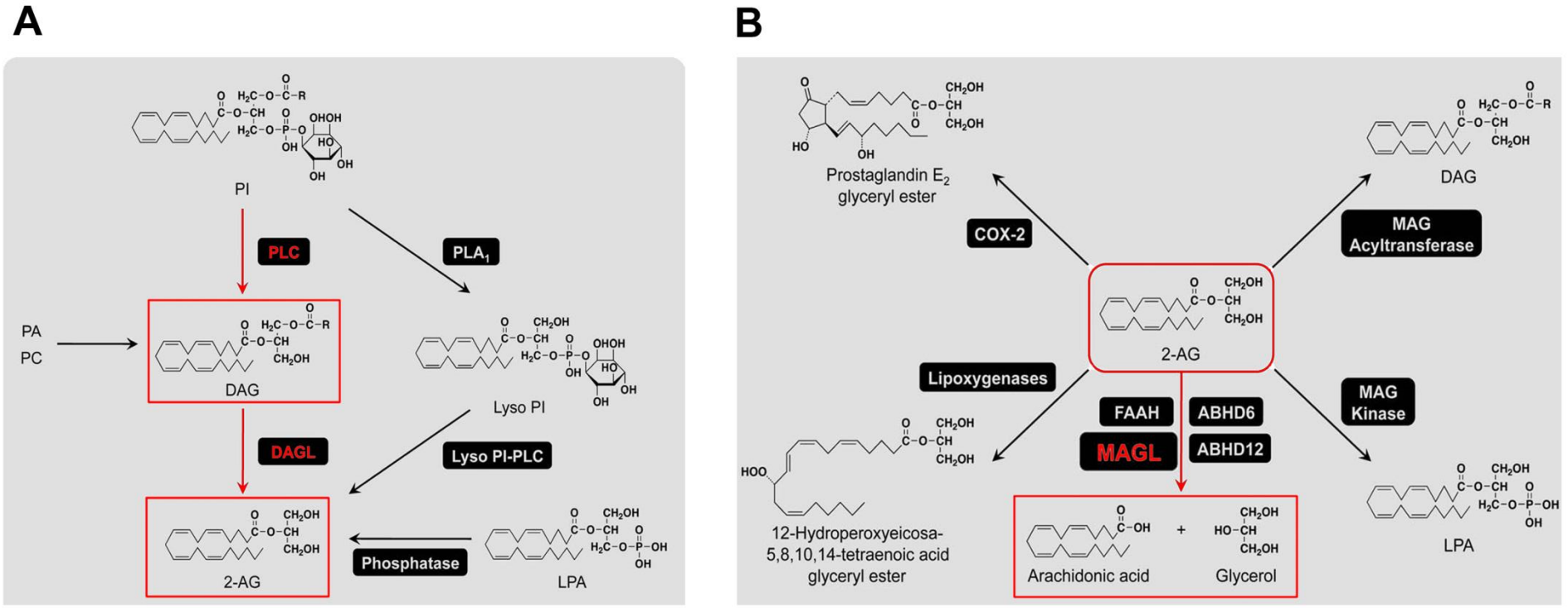
A
PI
PLC
PLA1
PA
PC
DAG
Lyso PI
DAGL
Lyso PI-PLC
2-AG
Phosphatase
LPA
B
Prostaglandin E2
glyceryl ester
COX-2
DAG
MAG
Acyltransferase
2-AG
Lipoxygenases
FAAH
ABHD6
MAGL
ABHD12
MAG
Kinase
12-Hydroperoxyeicosa-
5,8,10,14-tetraenoic acid
glyceryl ester
Arachidonic acid
+
Glycerol
LPA

Figure 1-1 The main synthetic and degradative pathways of 2-AG

The main pathways for 2-AG synthesis and degradation are shown above. (A) In the CNS, PLC synthesizes DAG from PI and DAGL then generates 2-AG from DAG (pathway shown in red). 2-AG can also be produced from phosphatidic acid (PA) and phosphatidylcholine (PC) by the action of DAGL or by sequential hydrolyses of PI via Lyso PI, 2-arachidonoyl LPA can also be converted to 2-AG via a phosphatase, but the relative importance of these pathways is less clear. (B) 2-AG is mainly hydrolysed to AA and glycerol by MAGL and this pathway is considered to be responsible for ~85% of the 2-AG hydrolysing activity in the brain (pathway shown in red). α and β-hydrolase domain-containing protein-6 (ABHD6) and ABHD12 and fatty acid amide hydrolase (FAAH) can also hydrolyze 2-AG in the brain. Adapted from Ueda *et al* (Ueda et al, 2011).

1.2 Functions of the eCB system in the developing CNS

1.2.1 Axonal growth and guidance

Axonal growth and guidance is the process by which neurons send out their axons to reach their correct targets. In the developing CNS, axons often manage to find their accurate paths and the underlying mechanisms are not fully understood (Bouquet & Nothias, 2007). The eCB signalling has been reported to be implicated in axonal growth and guidance in several studies (Berghuis et al, 2007; Keimpema et al, 2011; Watson et al, 2008; Williams et al, 2003; Wu et al, 2010). For example, consumption of cannabis during pregnancy and adolescence can cause damage to brain development and cognition (Spano et al, 2007). Prenatal THC exposure leads to over-activation of the CB1 receptor, affecting the proper positioning of cortical neurons in the developing cortex. Indeed, expression of the CB1 receptor has been detected in the human foetal brain and prenatal cannabis exposure can complicate in utero development of the nervous system and lead to neurobehavioral and cognitive defects (Berghuis et al, 2007; Keimpema et al, 2011; Wang et al, 2003). These findings together suggest that the eCB system might be involved in the forming of correct neuronal circuits during CNS development.

The distribution pattern of the CB1 and CB2 receptors in the developing brain is highly correlated with the expression of the DAGLα/β enzymes, the enzymes responsible for 2-AG synthesis (Berghuis et al, 2007; Bisogno et al, 2003; Mulder et al, 2008; Oudin et al, 2011b), suggesting that locally synthesised 2-AG is able to trigger signalling via closely located CB receptors. For example, 2-AG synthesised by DAGL activates the CB1 receptor in the same presynaptic growth cone of developing axons to promote axonal growth from cultured neurons (Williams et al, 2003). The CB1 receptor is reported to be enriched in axonal growth cones of gamma-aminobutyric acid-containing (GABAergic) interneurons during rodent cortex development, when the CB1 receptor is specifically deleted from these neurons, the target selection is impaired (Berghuis et al, 2007). In another study, both genetic and pharmacological ablation of the CB1 receptor leads to axon fasciculation deficits of pyramidal neurons in embryonic and early postnatal mice (Mulder et al, 2008). DAGLs are expressed by pyramidal cells and targeted to the axon with DAGLα exhibiting a proximal localization to the CB1 receptor in elongating axon shafts and growth cones, while MAGL was limited to axonal shafts, suggesting an intrinsic eCB tone within the growth cone is required to initiate axonal elongation and polarization during development (Mulder et al, 2008). Moreover, DAGL-dependent eCB signalling has been shown to be an important modulator for

corticothalamic and thalamocortical axon fasciculation (Wu et al, 2010). All these data together support the role of the eCB signalling in axonal growth and guidance during brain development. Changes in the eCBs signalling can significantly affect brain development and impair cognitive performance by affecting correct axonal targeting and fasciculation. The key question is how does the eCB signalling couple to other signalling cues to regulate axonal growth and guidance?

Axonal outgrowth and guidance is controlled by specific extracellular cues which are diffusible or bound to extracellular matrix or cell membranes (Bouquet & Nothias, 2007). Previous work in our lab has shown that cell adhesion molecules (CAMs) such as neural CAM (NCAM), N-cadherin and L1 play important roles in promoting axonal growth (Cavallaro & Dejana, 2011; Doherty et al, 1990; Walsh & Doherty, 1997; Walsh et al, 1997; Williams et al, 2003). They can act as surrogate ligands for the FGFR (fibroblast growth factor receptor) to trigger subsequent signal cascades that allows calcium influx into the growth cones to promote axonal growth and guidance (Williams et al, 2003). FGFR activated by CAMs can stimulate a phospholipase C-γ (PLCγ)-dependent synthesises of DAG, the substrate of the DAGLs (Hall et al, 1996b). DAGL couples the FGFR-PLCγ pathway to an axonal response by stimulating calcium influx into the growth cone through N- and L-type calcium channels (Doherty et al, 2000; Williams et al, 1994a; Williams et al, 1994b). Inhibition of DAGL can abolish neurite outgrowth stimulated by CAMs. Also the CB1 receptor is shown to function downstream from the FGFR activation, but upstream from calcium influx into cells (Williams et al, 2003). Inhibiting DAGL significantly reduces axonal outgrowth stimulated by N-cadherin or FGF-2 in developing Xenopus retinal ganglion cells (RGCs) (Lom et al, 1998). Together these data support the hypothesis that activation of FGFR signalling can lead to activation of PLCγ to synthesize DAG, DAGL then uses this substrate to generate 2-AG to activate the CB1 receptors in an autocrine fashion, which in turn leads to calcium influx via L- and N-type calcium channels to promote axonal growth (Williams et al, 2003) (A model of this pathway is shown in Figure 1-2).

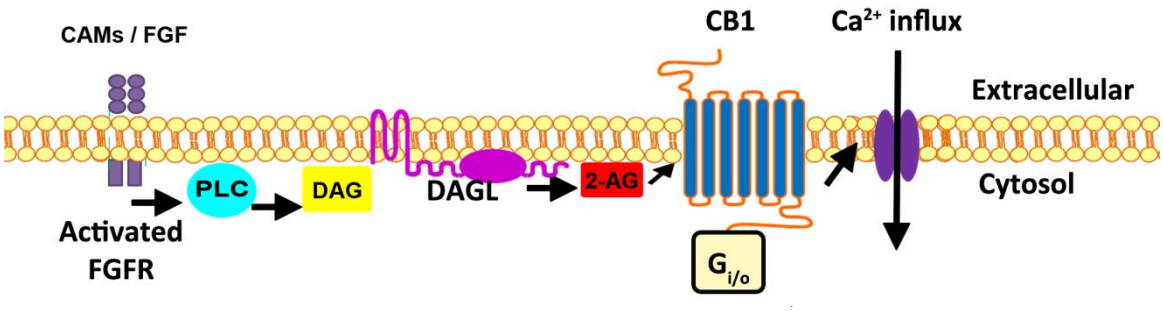

Figure 1-2 DAGL-dependent eCB signalling in axonal growth

A simplified model showing DAGL-dependent eCB signalling in axonal growth. This model suggests that molecules like CAMs or FGFs can activate FGFRs, which in turn activate PLCγ to synthesize DAG. DAGL would then generate the eCB 2-AG from DAG, which would be released to activate the CB receptors. Activation of CB receptors would lead to Ca^{2+} influx via N- and L-type voltage gated Ca^{2+} channels into the cell, which would induce axonal growth. Adapted from (Williams et al, 2003; Zhou et al, 2014).

1.2.2 Signalling pathways cross talk to the eCB system: FGFR and BDNF signalling

Besides the important roles in axonal growth and guidance, eCB signalling is also involved in many other physiological processes such as neurogenesis, synaptogenesis and the migration of neurons and neural progenitors of the developing and postnatal brain (Gao et al, 2010; Goncalves et al, 2008; Oudin et al, 2011a; Oudin et al, 2011b; Zhou et al, 2015). The wide variety of roles of the eCB system in the CNS is due to its ability to trigger or couple to downstream/upstream signalling pathways. CB receptors are GPCRs which are able to trigger a large number of downstream events, including regulation of the PI3K and MAPK pathways, cAMP and intracellular calcium levels, small GTPase-dependent cytoskeletal rearrangement upon stimulation (Zhou et al, 2014). However, these signalling pathways, together with the pathways upstream of the eCB signalling are mainly uncharacterized. Studies in our lab and some other studies suggest that the FGFR and BDNF signalling are two possible candidates that drive the eCB signalling during development to promote axonal growth (Aso et al, 2008; Berghuis et al, 2005; Doherty et al, 2000; Hall et al, 1996a; Williams et al, 2003).

As mentioned above, studies in axonal growth and guidance suggest that FGFR signalling can cross talk to the eCB signalling by activating PLCγ and subsequently make 2-AG by DAGL to activate CB receptors (Williams et al, 2003). Like the eCB signalling, FGFR signalling also plays important roles in the CNS such as neurogenesis and neuronal migration. For example, cells within the subventricular zone (SVZ) express FGFR and its ligand FGF-2, FGF-2 knock-out in mice can reduce the SVZ proliferation levels and the number of cells migrating to the olfactory bulb (OB) by ~50% (Zheng et al, 2004). Likewise, pharmacological inhibition of DAGL or genetic deletion of DAGLα in mice leads to ~50% reduction in SVZ neurogenesis (Gao et al, 2010; Goncalves et al, 2008). Both FGF-2 and CB receptor agonist can promote neuroblast migration out of the explant derived from the rostral migratory stream (RMS) (Garcia-Gonzalez et al, 2010; Oudin et al, 2011a) (details on SVZ neurogenesis and neuroblast migration in the RMS will be discussed in the next section). Taken together, FGFR signalling can couple to the eCB system during axonal growth and guidance; it might also cross talk to the eCB signalling on SVZ neurogenesis and neuroblast migration.

BDNF and its receptor TrkB have been shown to be able to cross talk to the eCB signalling in several studies (Aso et al, 2008; Berghuis et al, 2005; Blazquez et al, 2015; Lemtiri-Chlieh & Levine, 2010; Maison et al, 2009). For example, local administration of BDNF in the hippocampus

was able to reverse the anxiety-like phenotype seen in the CB1 receptor knock-out mice (Aso et al, 2008). BDNF can increase the expression of the CB1 receptor by increasing gene transcription and thereby increase neuronal sensitivity to 2-AG in cultured cerebellar granule neurons (CGNs) (Maison et al, 2009). BDNF also mediates the CB1 receptor function in mouse striatum. A single intracerebroventricular injection of BDNF *in vivo* was able to alter the activity of a subset of the CB1 receptor controlling GABA synapses in the striatum (De Chiara et al, 2010). The BDNF-TrkB signalling pathway has also been shown to be able to trigger eCB release at inhibitory synapses in the neocortex (Lemtiri-Chlieh & Levine, 2010). There is also evidence that the eCBs can use TrkB receptor-dependent signalling pathways to regulate interneuron migration (Berghuis et al, 2005) (details of the BDNF and eCB on cell migration will be discussed in the next section). More recently, a study reported the interaction of BDNF with eCBs accounts for the regulation of cocaine-induced synaptic plasticity in mouse dopaminergic neurons (Zhong et al, 2015). Another recent study reported that injection of a viral vector expressing the CB1 receptor into the dorsolateral striatum of R6/2 mice, a well-established model of Huntington's disease (HD), rescued the expression of BDNF as well as the HD-like molecular-pathology markers in these animals, suggesting an important role of the CB1/BDNF axis in disease (Blazquez et al, 2015).

Together, both FGFR and BDNF are able to cross-talk with eCB signalling during brain development. FGFR couples to the eCB system to regulate axonal growth and guidance, while a wide range of mechanisms can account for the eCB/BDNF cross-talk. Other signalling pathway such as epidermal growth factor receptor (EGFR) may also be able to drive the eCB signalling in the CNS (Cipriano et al, 2014; Sutterlin et al, 2013). However, further studies will be required to identify other signalling pathways and understand the underlying mechanisms of the eCB signalling network in the CNS.

1.3 Neurogenesis and neuronal migration

1.3.1 Neurogenic niches in the CNS-studies implicating eCBs

Neurogenesis is a process of generating functional neurons from neural stem cells and neural progenitors (Ming & Song, 2011). Neurogenesis is most active during prenatal CNS development to populate the growing brain with neurons. However, this process is restricted after birth in the mammalian brain, including humans (Ming & Song, 2011; Sanai et al, 2011). It is now widely accepted that in almost all mammals, active adult neurogenesis occurs throughout life in mainly two brain regions, the SVZ of the lateral ventricles and the subgranular zone (SGZ) in the dentate

gyrus of the hippocampus (Kazanis, 2013; Ming & Song, 2011). Altman, whose pioneering studies conducted in the 1960s discovered postnatal rat hippocampus neurogenesis, first suggested that new neurons are critical for learning and memory (Altman & Das, 1965; Altman & Das, 1967). In the past few years, considerable evidence has been obtained that supports a critical role of adult neurogenesis in hippocampal and olfactory bulb memory functions (Deng et al, 2010; Lazarini & Lledo, 2011; Ming & Song, 2011). Importantly, adult neurogenesis also holds therapeutic potential because of its relationship with degenerative neurological disorders, and the ability of neuroblasts to migrate to the site of brain injury and possibly replace dying neurons (Arvidsson et al, 2002; Curtis et al, 2009; Emsley & Hagg, 2003; Sundholm-Peters et al, 2005).

The SVZ is one of the crucial neurogenic niches in the brain where thousands of neural precursors are created every day in the SVZ of postnatal and adult mammalian brains. SVZ-derived neuroblasts then migrate in chains to the OB via the RMS, making it one of the longest migration process in the postnatal and adult brain (Figure 1-3B) (Doetsch & Alvarez-Buylla, 1996; Ming & Song, 2005; Ming & Song, 2011). Studies on postnatal and adult neurogenesis in the SVZ and migration along the RMS in the recent years have become a useful system in revealing the potential molecular mechanisms involved in neurogenesis and neuronal migration (Figure 1-3B) (Ming & Song, 2005; Ming & Song, 2011). Many proteins and signalling pathways, such as adhesion molecules (PSA-NCAM, integrin), extracellular cues (NRGs, Slits), Notch receptors, Ephrins and neurotrophins have been found to contribute to neurogenesis and migration in the SVZ and RMS. Some of these pathways are also conserved in embryonic development, but the whole system is far from been fully understood (Ming & Song, 2011). Additionally, some studies have suggested that the progenitor cells produced by the SVZ can migrate out of the RMS to the site of brain injury (Arvidsson et al, 2002; Curtis et al, 2009; Emsley & Hagg, 2003; Goings et al, 2004; Gotts & Chesselet, 2005b; Romanko et al, 2004; Sundholm-Peters et al, 2005). It is therefore fundamental to fully understand how migration is regulated in the RMS for potential therapeutic applications.

The eCB system has been shown to play a role in regulating proliferation of neural progenitors in both the adult hippocampus and SVZ and regulating neuroblast migration in the RMS (Gao et al, 2010; Goncalves et al, 2008; Oudin et al, 2011a). DAGL-dependent eCB signalling regulates adult SVZ neurogenesis in an age-dependent manner (Goncalves et al, 2008). Both hippocampal and SVZ neurogenesis were compromised in DAGL knock-out mice and there was ~50%

reduction of SVZ neurogenesis in DAGLα knock-out mice (Gao et al, 2010), suggesting the important role of the eCB signalling in maintaining the function of neurogenic niches. Also RMS neuroblasts express several components of the eCB system including DAGLα, MAGL and the CB1 receptor, supporting their ability to synthesise 2-AG and at the same time respond to the CB receptor signalling (Oudin et al, 2011b). Studies on cultured neuroblasts have clearly shown that activation of the CB receptors promotes neuroblast migration, facilitating the cycling between leading process extension and forward nuclear movement (Oudin et al, 2011b). Inhibition of eCB signalling instead causes shortening and branching of the leading process *in vitro* and *in vivo*, and this is associated with reduced migration from SVZ explants (Oudin et al, 2011b). Taken together, the evidence gathered from these studies identifies a fundamental role for eCB signalling in neurogenesis and neuronal migration in the mammalian brain; however the studies on migration were largely restricted to *in vitro* studies.

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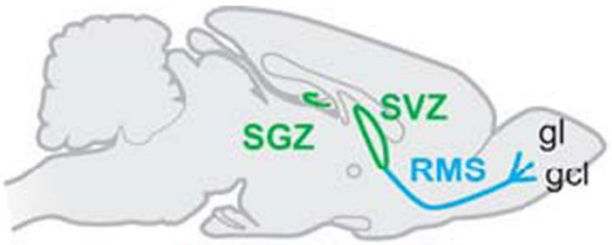
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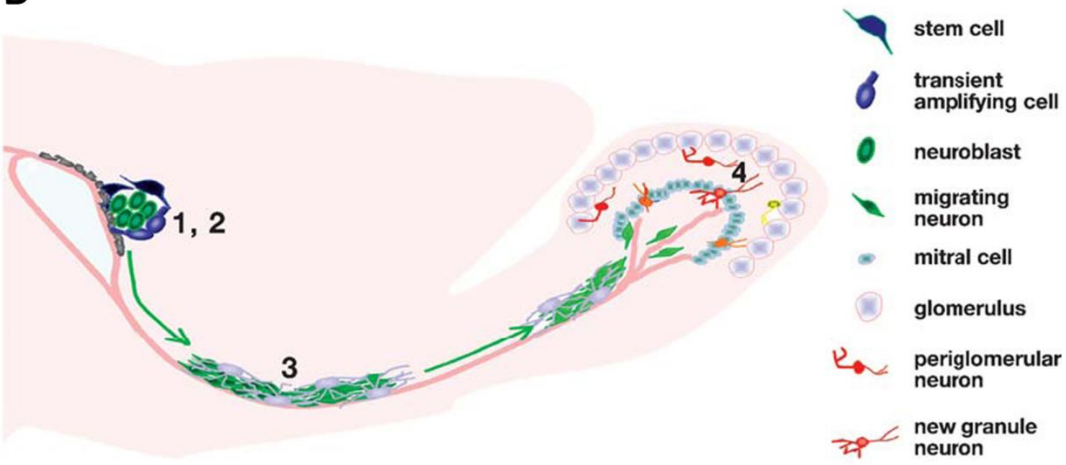
B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migrating neuron
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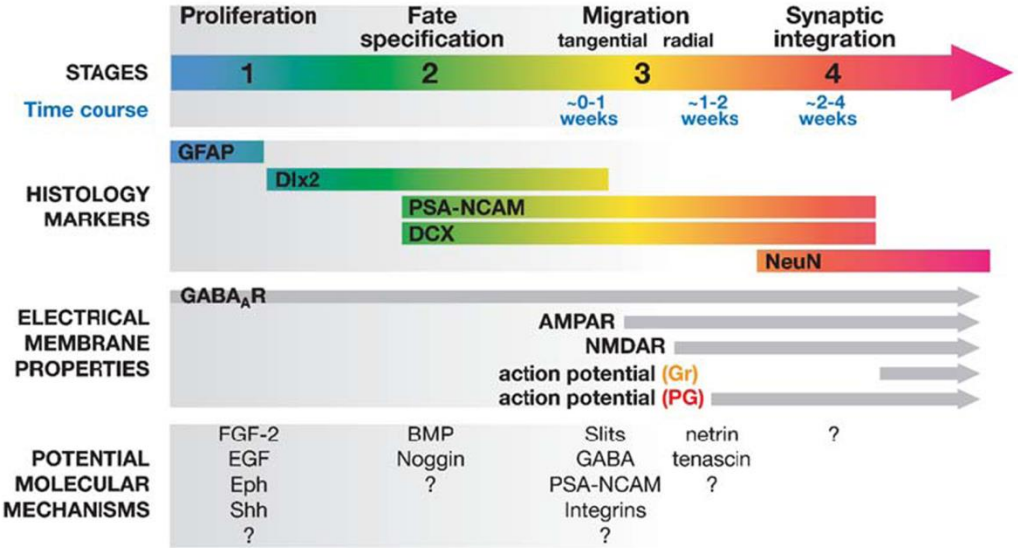
glomerulus
periglomerular neuron
new granule neuron


Proliferation
Fate specification
Migration
tangential
radial
Synaptic integration
STAGES
1
2
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Time course
~0-1 weeks
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~2-4 weeks
HISTOLOGY MARKERS
GFAP
Dlx2
PSA-NCAM
DCX
NeuN
ELECTRICAL MEMBRANE PROPERTIES
GABA$_A$R
AMPAR
NMDAR
action potential (Gr)
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POTENTIAL MOLECULAR MECHANISMS
FGF-2
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Shh
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BMP
Noggin
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Slits
GABA
PSA-NCAM
Integrins
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tenascin
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Figure 1-3 Neurogenic niches and SVZ neurogenesis in the postnatal and adult rodent brain

A sagittal section view of a postnatal adult rodent brain highlighting the two restricted regions that exhibit active adult neurogenesis: dentate gyrus (DG) in the hippocampal formation (HP), and the subventricular zone (SVZ) of the lateral ventricle (LV) (A). (B) Summary of four developmental stages during adult SVZ neurogenesis: Stage 1. Proliferation: stem cells (blue) in the SVZ of the lateral ventricles give rise to transient amplifying cells (light blue). Stage 2. Fate specification: transient amplifying cells differentiate into immature neurons (green). Adjacent ependymal cells (gray) of the lateral ventricle are essential for neuronal fate specification by providing inhibitors of gliogenesis. Stage 3. Migration: Immature neurons (green) migrate with each other in chains through the RMS to the OB. The migrating neurons are ensheathed by astrocytes. Once reaching the bulb, new neurons then migrate radially to the outer cell layers. Stage 4. Synaptic integration: Immature neurons differentiate into either granule neurons (orange) or periglomerular neurons (red). Potential molecular mechanisms of each stage are summarized at the bottom, mainly on the basis of studies on adult mice. Adapted from Ming and Song (2005).

1.3.2 What regulates neuroblast migration in the RMS?

The migration of neural progenitors and newborn neurons represents a fundamental process for brain development. Remarkably, the migration of niche-derived neural progenitors persists after birth, and is essential for proper maturation and integration of newborn neurons into the pre-existing synaptic circuits (Belvindrah et al, 2011). Two classical types of migration have been identified in the CNS: radial and tangential migration (perpendicular and parallel to the pial surface, respectively) (Metin et al, 2008). These two types of migration are generally regarded to be operated by distinct cellular mechanisms; however, neuroblasts may alternate from radial to tangential movement and vice versa during their course of migration, suggesting that both types of migrations share common principles (Marin et al, 2010; Shinohara et al, 2012). For example, in postnatal rodent brain, SVZ-derived neuroblasts first migrate tangentially in chains from the SVZ to OB along the RMS, after reaching the OB, they switch to radial migration from the OB core towards different OB layers where they differentiate into interneurons (Doetsch & Alvarez-Buylla, 1996; Ming & Song, 2011).

Migratory neuroblasts in the RMS usually have a characteristic elongated cell body and a single leading process oriented toward the OB (Nam et al, 2007). They move towards the bulb in a unique form of collective migration by sliding over each other therefore this form of migration is not guided by radial glial or axonal fibres (Lois et al, 1996). As mentioned above, SVZ-derived neuroblasts migrating along the RMS to the OB is one of the longest migration routes in the CNS after birth. Also the RMS is a tortuous route which dips and curves. Therefore it's very unlikely that the migration of the neuroblasts is controlled by a single factor or molecular pathway. Indeed, research carried out in the last decade has identified many factors and signalling pathways that contribute to the migration process in the RMS. These include architectural guides, growth factors (FGF, BDNF, GDNF), adhesion molecules (PSA-NCAM, integrin) and extracellular cues (NRGs, Slits) (Anton et al, 2004; Bolteus & Bordey, 2004; Cavallaro & Christofori, 2004; Chazal et al, 2000; Chiaramello et al, 2007; Mobley & McCarty, 2011; Nguyen-Ba-Charvet et al, 2004; Sawamoto et al, 2006). However, the whole system is far from being fully understood (Ming & Song, 2011). Here we give a comprehensive but not exhaustive list of key guidance cues that have been implicated in the regulation of neuroblast migration in the postnatal RMS.

1.3.2.1 Architectural guides

The flow of cerebrospinal fluid (CSF)

The CSF is a colourless body fluid produced in the choroid plexuses of the ventricles of the brain which acts as a basic mechanical and immunological protection to the brain. Coordinated, whiplike motion of ependymal cilia is required for normal CSF flow (Sawamoto et al, 2006). In *Tg737*^{orpk} mutant mice which have fewer, shorter and irregular cilia that are usually not motile, lack of normal CSF flow was observed (Sawamoto et al, 2006). PSA-NCAM staining showed that the migratory chains are disoriented in the SVZ in these mutant mice, and significantly less cells reach the OB compared to the wild type (WT) animals (Sawamoto et al, 2006). Using cell transplantation to graft mutant SVZ cells into WT mice, the authors found that the cells can migrate to the OB as normal, therefore it is not the mutant cells themselves that disrupt the migration. When grafting WT cells into mutant mice, the cells cannot migrate properly. Also the gradient of Slit2, which acts as a chemorepulsive factor for neuroblast migration (Hu, 1999), is absent in the mutant mice, suggesting that normal CSF flow is essential for the formation of a chemorepulsive factor gradient that guides directed neuroblast migration at the beginning of the RMS (Sawamoto et al, 2006). However, the neuroblasts migrated normally to the bulb once they managed to enter the stream despite the loss of the chemorepulsive gradient (Sawamoto et al, 2006), indicating the existence of other guidance cues in the SVZ and RMS.

Astrocytes

Neuroblasts migrating in chains in the RMS are ensheathed by astrocytes which form glial tubes to support migration (Lois et al, 1996). This type of tangential migration within a glial tube is 20% faster than migration as individual cells, suggesting that the astrocytes might be one of the guidance cues for migration in the RMS (Bovetti et al, 2007). Indeed, knock-out mice studies have shown that beta1 class integrins are required for the maintenance of the glial tubes and, defects in the glial tubes lead to the ectopic migration of neuroblasts into the surrounding tissue (Belvindrah et al, 2007). Therefore, the astrocytes within the RMS seem to create a path to facilitate neuroblast migration and serve as a physical barrier. However, astrocytes are not required for the chain migration of neuroblasts *in vitro* such as neuroblast migration out of the RMS-derived explant cultures (Cayre et al, 2009; Wichterle et al, 1997), indicating the importance of other guidance cues for migration.

Electric currents

A recent study reported that naturally occurring electric currents flow from the SVZ to the OB and these endogenous electric currents serve as a guidance cue for neuroblast migration (Cao et al, 2013a). By using the vibrating probe system, the authors detected an inward electric current of 1.6±0.4 μA/cm^2 and an outward electric current of 1.5±0.6 μA/cm^2 at the LV wall and the surface of OB, respectively (Cao et al, 2013a). Na+/K+ -ATPases distributed at the basal side of the LV wall and the apical side of epithelial layer of OB are likely to be responsible to generate this 3-5 mV/mm voltage along the RMS pathway from the SVZ to the OB (Cao et al, 2013a). Moreover, applied electric fields of similar strength direct migration of neuroblasts from the SVZ in culture and in brain slices (Cao et al, 2013a). The authors also showed that the purinergic receptor P2Y1 that are expressed transiently in the SVZ neuroblasts seem to mediate this electric field guided migration (Cao et al, 2013a). Therefore these data suggest that the naturally occurring electric potential gradients are a long-distance directional signal for neuroblast rostral migration (Cao et al, 2013a).

1.3.2.2 Growth factors

FGF-2

FGF-2 and the FGFRs play important roles in cell proliferation and neurogenesis in the SVZ (Frinchi et al, 2008; Mudo et al, 2007). FGF-2 is expressed in GFAP positive cells and may act on the FGFR1 expressed in nestin positive neural stem cells in the SVZ (Belluardo et al, 2008; Mudo et al, 2007). The role of FGF-2 on neuroblast migration has also been reported (Garcia-Gonzalez et al, 2010). Treatment of SVZ derived explant cultures with FGF-2 significantly increased migration out of the explants, and this effect was inhibited by the selective FGFR1 blocker, SU5402, suggesting an important role of FGF-2 and FGFR1 on neuroblast migration (Garcia-Gonzalez et al, 2010). Moreover, an immunostaining study revealed a transient caudal-rostral expression gradient of FGF-2 along the RMS in postnatal rodent brain slices, with stronger labelling in the SVZ region than the OB (Garcia-Gonzalez et al, 2010). This caudal-rostral gradient of FGF-2 in the neuroblast migration route in the RMS might serve as a guidance cue to drive neuroblast migration in the RMS (Garcia-Gonzalez et al, 2010).

BDNF

BDNF signalling through the TrkB receptor plays an important role in controlling tangential interneuron migration in the developing cerebral cortex (Polleux et al, 2002). BDNF is also

involved in SVZ neurogenesis in the mammalian brain (Bath et al, 2012). Both BDNF and TrkB have been shown to be expressed throughout the SVZ-RMS-OB route *in vivo*, and TrkB is expressed by the migrating neuroblasts in the RMS (Bath et al, 2008; Chiaramello et al, 2007; Snapyan et al, 2009), suggesting a role for BDNF signalling in regulating the neuroblast migration in the RMS. By using *in vitro* explant cultures, BDNF has been demonstrated to promote SVZ derived neuroblast migration, acting both as inducer and attractant through TrkB activation (Chiaramello et al, 2007). Blocking BDNF function by an antibody has been reported to increase the speed of neuroblast migration without disrupting its guidance in the RMS (Bagley & Belluscio, 2010). However, another study reported that inhibiting BDNF signalling with a TrkB-Fc impairs neuroblast migration as well as directionality within the RMS in acute brain slices (Snapyan et al, 2009), also pharmacological blockade of BDNF-dependent pathways on SVZ explants *in vitro* impairs neuronal migration (Chiaramello et al, 2007). In addition, endothelial cells of blood vessels which outline the neuroblast migratory route in the RMS synthesize BDNF that fosters neuroblast migration via p75NTR expressed on neuroblasts (Snapyan et al, 2009). Together, these studies strongly suggest that BDNF signalling plays an important role in regulating neuroblast migration in the RMS by acting on multiple pathways.

Other growth factors

Apart from FGF-2 and BDNF, other growth factors such as GDNF (Paratcha et al, 2006), HGF (Garzotto et al, 2008) and VEGF (Wittko et al, 2009) have been reported to be involved in regulating neuroblast migration in the RMS.

1.3.2.3 Adhesion molecules

Since neuroblasts migrate in chains by sliding over each other in the RMS, it is not surprising to find that adhesion molecules such as the polysialylated form of NCAM (PSA-NCAM) and integrins which are located on the cell surface play important roles in regulating neuroblast migration. Both PSA-NCAM and integrins are expressed by the neuroblasts in the RMS (Belvindrah et al, 2007; Moraes et al, 2009). Enzymatic removal of polysialic acid (PSA) from the neuroblasts not only disrupts the tangential migration and cellular organization of the RMS, but also causes a massive dispersion of neuroblasts into the surrounding brain regions including the cortex and striatum (Battista & Rutishauser, 2010). Studies on genetically modified mice demonstrated that beta 1 integrins promote the formation of cell chains and are required for the maintenance of the glial tubes in the RMS (Belvindrah et al, 2007). These studies support the important roles of adhesion

molecules in RMS neurolast migration by suggesting that PSA-NCAM may create a permissive environment for neuroblast migration, while integrins are responsible for the formation of migration chains in the RMS.

1.3.2.4 The eCB signalling

The eCB signalling system has been shown to be involved in neuronal migration in several studies. During brain development, active eCB signalling in the ventricular zone controls neural progenitor proliferation and radial migration of immature pyramidal cells to their appropriate location in the cortical layers (Mulder et al, 2008). Moreover, the eCB signalling regulates the radial migration of cortical neuron precursors derived from ganglionic eminences in the developing brain. This process ensures the targeting of neural precursors to their final location in the neocortex and hippocampus, where they differentiate into GABAergic interneurons (Berghuis et al, 2005). The CB1 receptor is highly expressed in a subpopulation of cholecystokinin (CCK)-containing inhibitory interneurons, which follow a complex radial and tangential migratory pattern across the telencephalon, ultimately reaching the hippocampus (Morozov et al, 2009). Recent studies in our lab have shown that an endogenous cannabinoid tone controls the polarized morphology and migration of postnatal RMS neuroblasts *in vitro* and *in vivo* (Oudin et al, 2011a). Direct activation of the CB receptors increases RMS neuroblast migration from explant cultures. Likewise, preventing 2-AG breakdown by a MAGL inhibitor also has similar effect, suggesting an important role for DAGL-dependent eCB signalling in the RMS (Oudin et al, 2011a). Activation of the CB1 receptor also promotes the interaction between protein kinase C (PKC) and fascin, an actin-bundling protein highly expressed in migratory neuroblasts, and regulating the balance between actin remodelling and cell adhesion, an essential aspect of efficient neuroblast migration (Sonego et al, 2013a). Thus the eCB signalling is important for maintaining the polarized morphology of the neuroblasts, possibly by affecting a range of cytoskeletal regulators, to facilitate efficient cell migration in the RMS.

1.3.2.5 Other guidance cues

Other signalling pathways such as Slit proteins, which serve as chemorepulsive factors, also contribute to regulating neuroblast migration in the RMS (Hu, 1999; Wu et al, 1999). In summary, the correct migration process of the neuroblasts from the SVZ to the OB requires the migrating cells be able to read or respond to multiple guidance cues and integrate these signals in a co-

ordinated manner. Further studies will be required to understand this integration and to perhaps draw a complete map on the regulation of the migration in the RMS.

1.4 Synaptic plasticity in the adult brain

1.4.1 Synaptic plasticity-roles of the eCB signalling

The role of the synapses in the adult brain is not only to transfer information from one neuron to another; they also have the capacity to change their strength through their activity over time. These changes may involve the strengthening or weakening of existing synapses as well as synapse formation and elimination. It is widely accepted that these activity-dependent changes in synaptic efficacy are necessary and sufficient for information storage in the brain. Therefore synaptic plasticity is thought to be the basis for learning and memory in the adult brain (Holtmaat & Svoboda, 2009; Martin et al, 2000; Neves et al, 2008).

One of the most important roles of the eCB signalling in the CNS is to act as a retrograde signalling system to regulate synaptic function (Castillo et al, 2012; Kano et al, 2009). Studies have shown that AEA and 2-AG can be released from postsynaptic neurons to act as retrograde messengers to target presynaptic CB receptors to suppress neurotransmitter release in either a transient or long-lasting manner, at both excitatory and inhibitory synapses in the adult brain (Alger, 2002; Alger, 2009; Castillo et al, 2012; Kano, 2014). Studies on knock-out animals also show that DAGLα, an enzyme required for calcium-dependent 2-AG production, is involved in both long-and short-term eCB-dependent synaptic plasticity (Gao et al, 2010; Tanimura et al, 2010; Yoshino et al, 2011). Therefore, the eCB signalling can regulate a wide variety of neural functions in the brain such as learning, cognition and feeding behaviors by modulating synaptic plasticity and serves as an emerging therapeutic target (Castillo et al, 2012). Moreover, the abundance of the eCB components in the brain also suggest that the eCB signalling is fundamental modulator in synaptic function (Castillo et al, 2012).

In general, synaptic plasticity can be divided into two forms: short-term synaptic plasticity which lasts from milliseconds to at most a few minutes, while long-term synaptic plasticity can last from minutes to hours, days, even life time (Citri & Malenka, 2008). There are many forms of short-term and long-term synaptic plasticity that have been discovered so far (Citri & Malenka, 2008). Here we are going to review two forms of long-term synaptic plasticity (long-term potentiation (LTP) and long-term depression (LTD)) and two forms of short-term synaptic plasticity (DSI and

DSE) respectively. Among these, LTP is the most extensively studied form of synaptic plasticity with no evidence for eCB signalling playing a major role; however eCB signalling has been reported to contribute to, or mediate LTD, DSE and DSI (Castillo et al, 2012; Chevaleyre et al, 2006; Kano, 2014; Wilson & Nicoll, 2001; Wilson & Nicoll, 2002).

LTP

LTP is a persistent strengthening of synapses based on recent patterns of activity, it was first observed by Terje Lømo who found long-lasting increases in efficiency of transmission at synapses on dentate granule cells induced by repetitive trains of stimuli to the perforant path in anaesthetized rabbits in 1966 (Lomo, 2003). This observation was further characterized in 1970s (Bliss & Lomo, 1973) and remains a popular research subject to this day. LTP has been most studied in the mammalian hippocampus, an area of the brain that is believed to be important for memory formation (Lynch, 2004). Here the axons of pyramidal neurons in the CA3 region of hippocampus form synapses in the CA1 region pyramidal neurons, these axons are called Schaffer collaterals. Electrical stimulation of Schaffer collaterals can generate excitatory postsynaptic potentials (EPSPs) in the postsynaptic CA1 neurons. If the stimulation is only two or three times per minute, the size of the evoked EPSP in the postsynaptic CA1 neurons remains relatively constant. However, strong tetanic stimulation (a brief, high-frequency sequence of individual stimulations) to the same axons causes a long-lasting increase in the postsynaptic EPSP amplitude, and this is LTP (Cooke & Bliss, 2005; Lomo, 2003; Lynch, 2004). LTP has been found to be input-specific, only activated synapses on a given cell are potentiated. It also has property of associativity. If two pathways converge upon the same postsynaptic neuron and one pathway has only modest compact on the postsynaptic neuron, this weak stimulation will not by itself trigger LTP. However, if this weak pathway is activated concurrently with the other strong pathway, then both pathways can be strengthened. These two properties of LTP are believed to serve as cellular basis for learning and memory such as associative learning and memory formation (Cooke & Bliss, 2005; Lynch, 2004).

LTD

LTD is another form of long-term synaptic plasticity. It is different from LTP, which occurs when the Schaffer collaterals (or other axons) are stimulated by high-frequency stimulation, in that LTD is stimulated by low rate stimuli (~1Hz) for long periods (10-15 min) and causes a long-lasting

reduction in the efficacy of synapses. However, like LTP, LTD is also activity-dependent and can last for a long period of time (Bol'shakov, 2001; Collingridge et al, 2010). These common features and complementarity of LTP and LTD suggest they reversibly affect synaptic efficacy to regulate synaptic plasticity in the CNS. LTD can selectively decrease synaptic strength and erase the increase in EPSP amplitude cause by LTP (Collingridge et al, 2010; Malleret et al, 2010). This function might be important for new information acquisition by clearing old memory traces, thereby preventing those traces from interfering with newly formed information (Malleret et al, 2010).

The molecular mechanisms of LTD are not entirely clear. Studies suggest that N-methyl-D-aspartate receptors (NMDARs) and metabotropic glutamate receptors (mGluRs) are important in mediating LTD function (Collingridge et al, 2010; Hu et al, 2014; Yashiro & Philpot, 2008). Neural activity from the presynaptic neurons usually triggers the activation of these postsynaptic receptors and calcium-dependent second messenger systems. These second messenger systems can then alter the activity of protein kinases or phosphatases to mediate the early stages of long-term synaptic plasticity. As we mentioned above, DAGL-dependent eCB signalling is able to release 2-AG in response to calcium influx (Bisogno et al, 2003). Also, eCB signalling has been shown to mediate presynaptic forms of LTD at both excitatory and inhibitory synapses in several brain regions (Castillo et al, 2012; Kano et al, 2009). Postsynaptic Group I mGluRs, PLC, and DAGLα are usually required for eCB-mediated LTD, 2-AG synthesized by DAGLα can then act on the CB1 receptor localized to excitatory or inhibitory synaptic terminals to suppress neurotransmitter release (Figure 1-4A). However, there is not a full consensus on eCB-mediated LTD as different results have been reported from different brain regions (Chevaleyre & Castillo, 2003; Kano et al, 2009). Therefore further studies will be required to fully understand the underlying mechanisms of eCB-mediated LTD and long-term synaptic plasticity.

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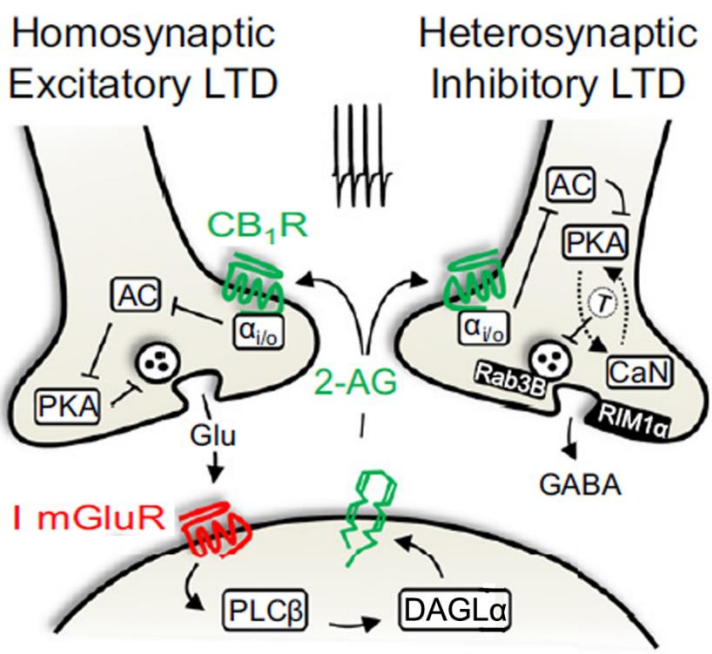

Homosynaptic
Excitatory LTD
Heterosynaptic
Inhibitory LTD
CB1R
AC
αi/o
PKA
Glu
2-AG
AC
PKA
T
αi/o
Rab3B
CaN
RIM1α
GABA
I mGluR
PLCβ
DAGLα

B

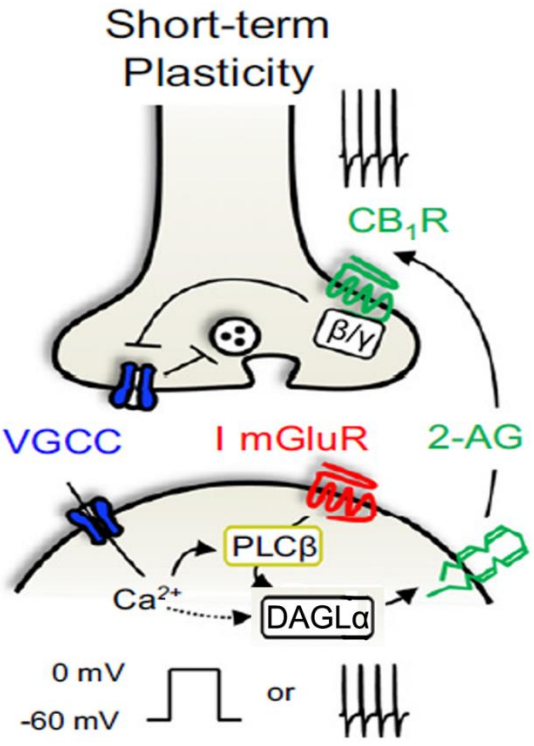

Short-term
Plasticity
CB1R
β/γ
VGCC
I mGluR
2-AG
PLCβ
Ca2+
DAGLα
0 mV
-60 mV
or

Figure 1-4 eCB-mediated long- and short-term synaptic plasticity

In the adult brain, DAGL expression is restricted to dendritic postsynaptic compartments to release newly synthesised eCBs acting as retrograde messengers on the presynaptic CB1 receptor, suppressing further transmitter release in excitatory and inhibitory synapses. (A) eCB-mediated excitatory and inhibitory LTD. Patterned stimulation releases neurotransmitter glutamate (Glu) at presynaptic sites, Glu activates postsynaptic mGluRs which couple to PLCβ and DAGLα. 2-AG synthesised by DAGLα then targets the CB1 receptor localized to excitatory or inhibitory synaptic terminals where $G_{i/o}$ protein coupled to the CB1 receptor can reduce adenylyl cyclase (AC) and protein kinase A (PKA) activity to suppress neurotransmitter release. (B) eCB-mediated short-term depression. Postsynaptic activity triggers calcium influx via voltage-gated calcium channels (VGCCs) and leads to DAGLα-mediated eCB mobilization. Presynaptic activity can also promote 2-AG production through DAGLα by activating mGluRs. 2-AG then acts as a retrograde messenger to target the presynaptic CB1 receptors to reduce neurotransmitter release. Adapted from Castillo *et al* (Castillo et al, 2012).

DSI/DSE

DSI and DSE are two closely related forms of short-term synaptic plasticity induced by depolarization of the post-synaptic site resulting in suppression of the pre-synaptic release of inhibitory or excitatory neurotransmitters mediated by the release of a retrograde messenger (Diana & Marty, 2004). DSI was first described in the early 1990s and many observations led to the conclusion that the phenomenon must involve retrograde signalling (Diana & Marty, 2004; Llano et al, 1991). However, the identity of the retrograde messenger mediating DSI and DSE remained unknown until 2001 when two groups reported that eCB signalling is responsible for mediating DSI in cultured hippocampal neurons and hippocampal slices (Ohno-Shosaku et al, 2001; Wilson & Nicoll, 2001). Shortly afterwards, the counterpart of DSI, eCB-mediated DSE for excitatory synaptic transmission in cerebellar Purkinje cells was also reported (Kreitzer & Regehr, 2001a; Kreitzer & Regehr, 2001b). As a matter of fact, these are the first discoveries of the eCBs acting as retrograde signalling molecules in the brain (Castillo et al, 2012; Kreitzer & Regehr, 2001a; Kreitzer & Regehr, 2001b; Ohno-Shosaku et al, 2001; Wilson & Nicoll, 2001). To date numerous studies have reported that a post-synaptically released eCB induces DSI and DSE in many different brain regions (Kano et al, 2009; Ohno-Shosaku et al, 2001; Pitler & Alger, 1992). Both DSI and DSE are calcium dependent and can be blocked by the CB1 antagonist AM251 (Kano et al, 2009). DSI can be induced only at cannabinoid-sensitive synapses, supporting the important role of eCB signalling in these two forms of synaptic plasticity (Kano et al, 2009; Ohno-Shosaku et al, 2001). Inhibition of MAGL, the enzyme responsible for 2-AG degradation, but not AEA hydrolyzing enzyme FAAH can generally prolong DSI and DSE (Hashimotodani et al, 2007). Moreover, two independent knock-out studies have shown that DSI and/or DSE are totally absent in the hippocampus, cerebellum and striatum in DAGLα (but not DAGLβ) knock-out mice (Gao et al, 2010; Tanimura et al, 2010), suggesting the important roles of DAGLα and 2-AG in mediating these two forms of synaptic plasticity. A model of eCB-mediated short-term synaptic plasticity is shown in (Figure 1-4B). The induction of DSI is triggered by elevation of postsynaptic calcium concentration. Calcium influx through voltage-gated calcium channels (VGCCs) or postsynaptic receptors like NMDA receptors may contribute to this process. This elevated calcium level leads to 2-AG production by DAGLα, 2-AG can then travel through the synaptic cleft by an unknown mechanism to activate the presynaptic CB1 receptors to reduce neurotransmitter release (Castillo

et al, 2012). The molecular mechanism of DSE on the other hand is less well understood (Diana & Marty, 2004).

It is worth noting that other types of eCB-mediated synaptic plasticity have also been reported. For example, calcium influx through NMDA receptor can directly induce eCB release, this process was abolished by blocking NMDA receptors, CB1 receptors and DAGL, but not by inhibiting VGCCs (Ohno-Shosaku et al, 2007). Presynaptic activity can also promote 2-AG production through DAGLα by activating mGluRs. It has been reported that activation of group I mGluRs is sufficient to mobilize eCBs to trigger both short- and long-term synaptic plasticity (Figure 1-4) (Castillo et al, 2012; Chevaleyre et al, 2006). The mechanisms of this receptor-driven eCB release are not entirely clear (Kano, 2014). However, data in this section point to the important role of postsynaptic DAGLα in the eCB-mediated synaptic plasticity. As we will discuss in detail in the next section, DAGLα may dynamically interact with postsynaptic receptors or other postsynaptic components to regulate synaptic plasticity in the brain.

1.4.2 Receptor endocytosis at the postsynaptic sites as a plasticity mechanism

Neurotransmitter receptors located at the postsynaptic sites are directly responsible for receiving the information released by presynaptic neurons, therefore the type, number and density of these receptors at a particular spine will affect the synaptic strength (Arancibia-Carcamo et al, 2006; Collingridge et al, 2004). Receptor endocytosis, an energy-using cellular process used by cells to internalize receptors and sort them inside of the cell through distinct endocytic pathways either for degradation or recycling, can powerfully control the number and density or even type of the receptors that appear on the cell membrane of the postsynaptic site (Arancibia-Carcamo et al, 2006; Collingridge et al, 2004). It has long been established that some of the most important postsynaptic receptors like AMPA, NMDA, GABA and mGluRs all undergo endocytosis and are dynamically cycled inside of the postsynaptic cells to regulate synaptic plasticity (Carroll et al, 1999; Dhami & Ferguson, 2006; Hardt et al, 2014; Kittler et al, 2005; Kittler et al, 2000; Scott et al, 2004; Yashiro & Philpot, 2008). Here we are going to review the endocytosis of those postsynaptic receptors to gain insights of this process in regulation of synaptic plasticity in the adult brain.

AMPA and NMDA receptors

AMPA and NMDA receptors are both ionotropic glutamate receptors that often co-exist at the same synapse. They are tetrameric complexes each consisting of four subunits (Karakas et al, 2015). When activated by glutamate, AMPA receptors react immediately to allow positively charged sodium ions to flow through the cell membrane thus mediate fast synaptic transmission (Gomes et al, 2003). NMDA receptors, on the other hand, mediate slow transmission because the pore of the receptor channel is blocked by a magnesium ion. This blockage is only broken when a certain level of depolarization is reached and AMPA receptors have allowed enough sodium to enter the cell membrane, thus this collection of positive charge will repel the magnesium ion out of the NMDA channels. When the NMDA receptors are open, they can actively respond to glutamate and allow not only sodium, but also calcium to enter the postsynaptic cell. The influx of calcium can then act as a second messenger to activate intracellular signalling cascades (Collingridge, 1987; MacDonald et al, 2006). For example, when a significant depolarization occurs at a postsynaptic site which induces rapid elevation of postsynaptic calcium, this intracellular calcium can interact with protein kinases such as PKC to phosphorylate target proteins which are able to regulate the trafficking of AMPA receptors. As a result, more AMPA receptors are inserted back to the postsynaptic membrane to make the terminal more responsive to glutamate. Thus this particular synapse is strengthened and this is believed to be the molecular mechanisms for LTP (Carroll et al, 2001; Gomes et al, 2003). In contrast, a low frequency stimulation which induces a modest increase of postsynaptic calcium can instead lead to activation of protein phosphatases which lead to dephosphorylation of target proteins and the internalization of AMPA receptors (Casimiro et al, 2011). Therefore less AMPA receptors are available at the postsynaptic site and the synapse is weakened. These processes serve as the molecular basis for another long-term synaptic plasticity, LTD (Gomes et al, 2003; Malenka, 2003).

Beyond the well-established role as triggers for LTP and LTD described above, NMDA receptors themselves are also dynamically regulated (Hunt & Castillo, 2012; Rebola et al, 2010). Increased α-synuclein expression, which is associated with neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD), has been shown to induce NMDA receptor internalization in cultured hippocampal neurons, as a result, the inward current and calcium influx upon NMDA receptor stimulation are reduced (Chen et al, 2015). In summary, the voltage-dependent property of the NMDA receptors and endocytosis of AMPA and NMDA receptors regulate the strength of the synapse and serve as molecular basis for learning and

memory and have been shown to be involved in many diseases such as epileptic seizures and depression (Casillas-Espinosa et al, 2012; Freudenberg et al, 2015).

mGluRs

Metabotropic glutamate receptors or mGluRs are 7-TM GPCRs. They can be classified into 3 groups based on receptor structure and physiological activity (Dhami & Ferguson, 2006). Different types of mGluRs are distributed differently in cells, group II and group III mGluRs are predominantly localized to presynaptic elements, while group I mGluRs are localized to postsynaptic sites (Shigemoto et al, 1997). Therefore, we are going to focus on group I mGluRs in this thesis. Group I mGluRs (mGluR1 and mGluR5) are GPCRs coupled to the heterotrimeric G protein alpha-q/11 and can act as modulators of other receptors (Dhami & Ferguson, 2006). For example, mGluR5 have been shown to be clustered to NMDA receptor-associated PDS-95 complex by Homer and Shank proteins, therefore they can be anchored close enough to NMDA receptors to modulate their activities (Tu et al, 1999). Indeed, activation of mGluRs and NMDA receptors has been shown to be able to trigger internalization of distinct populations of AMPA receptors in CA1 hippocampal neurons (Casimiro et al, 2011). Group I mGluRs can also regulate synaptic plasticity independent of NMDA receptors. A study reported that specific group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG) can induce depotentiation (the reversal of LTP) and trigger a protein synthesis-dependent internalization of synaptic AMPA receptors at Schaffer collateral–CA1 synapses of rat hippocampal slices (Zho et al, 2002).

Apart from modulating NMDA and other receptors, group I mGluRs also undergo endocytosis at postsynaptic sites themselves. For example, cycling of mGluR1 has been shown in both neuronal and non-neuronal cells (Pandey et al, 2014). In this study, the authors found that the majority of the mGluR1 receptors localized at the recycling compartment and can be recycled back to the cell membrane after been internalized. No significant presence of the receptor was noticed in the lysosome and the recycling of mGluR1 is protein phosphatase 2A dependent (Pandey et al, 2014). Studies on the molecular mechanisms of group I mGluRs have revealed that the endocytosis of these receptors can be both agonist-dependent and -independent (Dale et al, 2002). The regulation of group I mGluRs internalization appears to be mediated by similar molecular intermediates as have been described for more typical GPCR such as the beta(2)-adrenergic receptor (Dhami & Ferguson, 2006). Agonist-dependent mGluR internalization is mediated by clathrin and dynamin-dependent endocytic pathways (Dale et al, 2002). Upon activation, group I

mGluRs lead to the activation of PLCβ thereby stimulating IP3 and calcium release from intracellular stores and lead to further signal transduction (Dale et al, 2002). Referring to the DAGL signalling we mentioned above (Figure 1-1) and (Figure 1-4), we can see group 1 mGluRs and DAGLα converge at the postsynaptic sites. Indeed, uncoupling of mGluRs and DAGLα-dependent eCB signalling at the synapse has been implicated in abnormal LTD at excitatory synapses and the pathogenesis of Fragile X syndrome, a type of autism and most commonly inherited form of mental retardation in human (Dolen et al, 2007; Jung et al, 2012; Maccarrone et al, 2010). Studies from Fragile X syndrome animal models lead the authors to propose that mGluR5 receptors and DAGLα are linked together in a postsynaptic signalling complex to maintain normal state retrograde transmission at the synapse (Jung et al, 2012). Therefore DAGLα may be subject to endocytosis and recycling in a similar manner as group I mGluRs and thus may perhaps be considered as a new element of synaptic plasticity, albeit one that might simply be a mechanistic component of the well-established eCB pathway. This new idea will be explored in the second half of this thesis.

GABA receptors

The GABA receptors are a class of receptors that respond to GABA, the principal inhibitory neurotransmitter in the adult mammalian brain. There are two types of GABA receptors: ionotropic GABA(A) receptors which are ion channels and metabotropic GABA(B) receptors which are GPCRs. The GABA(A) receptors are the major site of fast synaptic inhibition in the CNS while the GABA(B) receptors mediate slow and prolonged inhibitory signals in the CNS (Kittler et al, 2000; Terunuma et al, 2010). Both GABA(A) and GABA(B) receptors have been shown to undergo clathrin-dependent constitutive endocytosis to regulate synaptic plasticity (Kittler et al, 2000; Terunuma et al, 2010). For example, inhibition of GABA(A) receptor internalization can enhance the amplitude of miniature inhibitory synaptic current and whole cell GABA(A) receptor current, indicating that the endocytosis of GABA(A) receptors regulate the efficacy of synaptic inhibition in hippocampal neurons (Kittler et al, 2005; Kittler et al, 2000). The GABA receptors can also couple to other signalling pathways such as endocytosis of AMPA receptors to regulate synaptic plasticity. For instance, a patterned high-frequency stimulation can trigger GABA(A) receptor-dependent LTD in rat hippocampus which is independent of NMDA receptors and requires the endocytosis of AMPA receptors (Zhu et al, 2013). Another study has reported the modulator effect of the GABA(B) receptors for mGluR1 in cerebellar Purkinje cells (Tabata & Kano, 2010). In these

cells, GABA(B) receptors co-localize with mGluR1 receptors around the postsynaptic membrane of the excitatory synapses to facilitate mGluR1-mediated synaptic plasticity like cerebellar LTP, which is crucial for cerebellar motor learning (Tabata & Kano, 2010).

In summary, receptor endocytosis at postsynaptic sites is crucial for the regulation of synaptic plasticity such as LTP, LTD and retrograde signalling, thus is important for learning and memory of the mature brain. Postsynaptic receptor endocytosis also has therapeutic importance as it is involved in diseases like PD, AD and Fragile X syndrome. Studies on receptor cycling have contributed to better understanding of underlying mechanisms of synaptic plasticity, however, it is still an ongoing process and further studies will be required. In the next section we will discuss some of the available tools for the studying of receptor endocytosis.

1.4.3 Tools for studying receptor endocytosis in neurons and non-neuronal cells

Endocytosis is a cellular process used by cells to internalize membrane proteins, lipids, extracellular ligands and soluble molecules (Arancibia-Carcamo et al, 2006). It is a basic cellular process that controls the protein and lipid composition of the plasma membrane therefore regulating how cells interact with and respond to their environment (Doherty & McMahon, 2009). The postsynaptic membrane is subjected to dynamic stimulation and is a site of highly regulated endocytosis in the CNS (Collingridge et al, 2004). A variety of endocytic pathways have been discovered and studied so far, however, these pathways are generally divided into two groups: clathrin-mediated endocytosis or clathrin-independent endocytosis. Clathrin-mediated endocytosis is the most extensively studied and best understood endocytic pathway. Clathrin is a protein that forms a triskelion shape by its heavy and light chain complex; this triskelion can assemble into an apolygonal lattice that helps to deform the plasm membrane into a clathrin-coated pit and subsequently into clathrin coated vesicles. AP-2 is required for the recruitment of clathrin to the plasma membrane and it provides a link between clathrin and the molecules that will be internalised via the clathrin coated pit (Farsad et al, 2003; Heilker et al, 1996; Traub, 2003; Traub, 2009). Dynamin is also essential for clathrin-coated vesicle formation as it is critical for the pinching and release of a completed vesicle from the plasma membrane (Doherty & McMahon, 2009). Once the clathrin built small vesicles detach from the plasma membrane, they are then transported within the cells. On the other hand, clathrin-independent pathways such as Caveolin and lipid raft-dependent pathways are less well understood (Doherty & McMahon, 2009; Mayor et al, 2014; Miaczynska & Stenmark, 2008). However, these clathrin-independent pathways have

been suggested to be important (or only functional) in special situations such as cell migration or in specific cell types like hippocampal neurons (Bitsikas et al, 2014; Doherty & McMahon, 2009; Howes et al, 2010; Mayor et al, 2014; Sandvig et al, 2011).

For both clathrin-dependent and -independent endocytosis pathways, once the receptors have been internalized, they enter the endocytic pathway which consists of distinct membrane compartments like endosomes or lysosomes (Arancibia-Carcamo et al, 2006). Studies on these intracellular compartments have revealed some important mechanisms in receptor intracellular trafficking and many important markers such as endosomal markers have also been identified (Arancibia-Carcamo et al, 2006; Barbieri et al, 1994; Bucci et al, 1992; Collingridge et al, 2004; Jovic et al, 2010; Mu et al, 1995). All of these findings have contributed to the development of new tools for receptor trafficking study. Here we are going to review some widely used tools that have been applied to both neurons and non-neuronal cells for receptor endocytosis study, some of these tools are used in this thesis for DAGL trafficking.

Antibody feeding

Antibody feeding is one of the key method that has been developed and widely used to study neurotransmitter receptor endocytosis and trafficking (Arancibia-Carcamo et al, 2006). It is an immunofluorescence labelling approach that involves using antibodies that recognize epitopes in the extracellular domains of the receptors being studied thus allowing the receptors to be visualized down to the endosomal pathway (Arancibia-Carcamo et al, 2006). Due to the increasing availability of fluorescent antibodies and the development in advanced microscopy and imaging techniques, this method has become really powerful in tracking the intracellular movement of the internalized receptors in either fixed or live cells.

The schematic steps of a typical antibody feeding assay are shown in Figure 1-5. Generally, the first step is antibody binding to the live cell: the receptor of interest can be labelled using an antibody against the extracellular epitope tag in live cells. This step is usually carried out at room temperature or at 4°C to limit receptor endocytosis during this first labelling step. After antibody binding, the cells are washing in PBS to remove unbound antibodies from the media leaving behind only the cell surface labelled receptors. Then the second step of the assay is internalization: cells are returned back to 37°C for the desired period of time in conditioned medium to allow receptor (and bound antibody) internalization. Cells are then placed on ice to

stop further endocytosis and are fixed without permeabilization. The third step is to label any antibody bound receptor that has remained at the cell surface with a fluorescent secondary antibody. Cells are permeabilized after this step, the fourth step is to label the internalized pool of the antibody/receptor complexes with another secondary antibody which is conjugated with a different type of fluorophore (Carrodus et al, 2014). Thus the surface pool and the internalized pool of the receptor are differentiated by different types of fluorophores, the rate of receptor internalization can then be quantified by comparing the signal ratio of these two fluorophores.

Modifications of the above steps are possible and variations of antibody feeding methods have been reported (Arancibia-Carcamo et al, 2006; Carrodus et al, 2014; Kennedy et al, 2010). For example, an antibody stripping method has been reported to be able to strip any remaining surface-bound primary antibody and leave intracellular antibody-receptor complexes intact by washing the cells at low pH prior to fixation (Arancibia-Carcamo et al, 2006; Hanley et al, 2002). This is extremely useful in studying postsynaptic receptor trafficking as extensive receptor expression at the cell surface could mask the signal for internalized receptors (Arancibia-Carcamo et al, 2006). Another study reported that an overnight blocking step can also be applied to distinguish surface and internalized receptors (Carrodus et al, 2014). In Figure 1-5C, it may happen that not all the surface receptors are labelled by the initial secondary antibody, these unlabelled receptors could be labelled by the subsequent secondary antibody at the later step thus make it difficult to differential the internalized receptors form the surface pool. For some experiments such as studies in cells lines this is not an issue as the rate of internalization can be calculated from the ratio of the fluorescence intensities normalized to untreated controls. However, for studies that requires higher resolution such as studies focusing on synapses and neuronal dendrites, this problem can be solved by applying an overnight blocking step with high-concentration of unlabelled secondary antibody between step C and D in Figure 1-5 (Carrodus et al, 2014). The antibody feeding technique can also be used in conjugation with time-lapse imaging to track the receptor endocytosis in a dynamic manner (Bien-Ly et al, 2014; Wirz et al, 2005). Overall, antibody feeding has been shown to be useful in obtaining information about the subcellular localization of a putative receptor in various cell types (Arancibia-Carcamo et al, 2006; Carrodus et al, 2014).

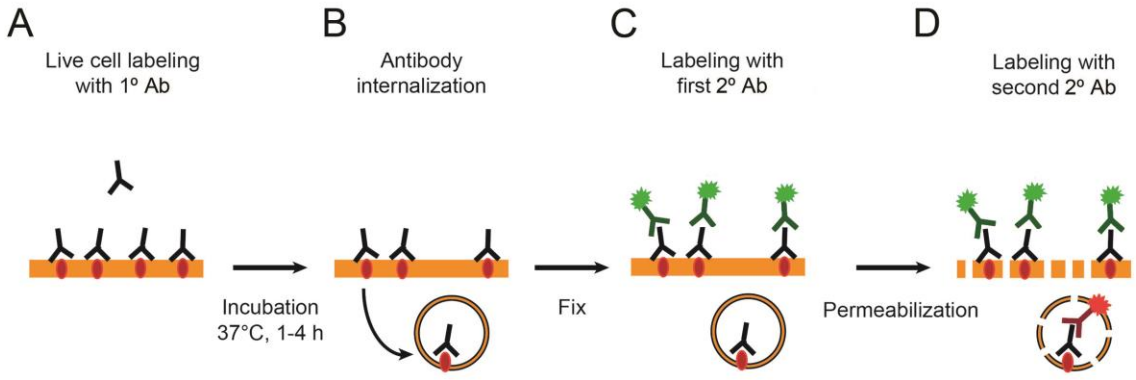


Figure 1-5 Schematic steps of antibody feeding assay

Cartoon showing an example of “antibody feeding” assay: In brief, live cultured cells were labelled for 20 min at room temperature (or 4°C) with an antibody directed against the extracellular tag (e.g. GFP) of the receptor (A). After washing in PBS to remove unbound antibodies from the cell surface, cells were incubated at 37°C in cell culture medium or medium containing pharmacological agents to allow for endocytosis (B). Cells were then fixed for 5 min at room temperature in 4% paraformaldehyde (PFA) without permeabilization, and stained with Alexa Fluor 488-conjugated secondary antibodies for 1 h at room temperature, to visualize surface receptors (C). Cells were then permeabilized for 10 min in 0.2% Triton X-100 in PBS containing 2% BSA and stained with Alexa Fluor 594-conjugated secondary antibodies for 1 h at room temperature in the same solution, to visualize internalized receptors (D). Fluorescence images can then be acquired using confocal microscopy. Units of internalization can be measured as red (indicative of internalization) / green fluorescence intensities normalized to untreated controls. Adapted from Carrodus *et al*. (Carrodus et al, 2014).

Cell surface biotinylation

Cell surface biotinylation is another widely used technique in studying receptor endocytosis. It is a biochemical method based on the covalent modification of surface receptors with biotin which enables all the cell surface proteins that have accessible lysine residues and sufficient extracellular exposure to be labelled at a particular time point. Treated cells can then be harvested and the subsequent fate of labelled surface proteins can be determined through affinity purification followed by SDS-PAGE and Western blotting (Arancibia-Carcamo et al, 2006). Cell surface biotinylation is becoming a popular technique due to the development of biotin related reagents and experimental kits. Although cell surface biotinylation does not allow visualization of the subcellular localization of the internalized receptors, it still has some advantages compared to the antibody feeding approach. First, the biotin molecule is much smaller than an antibody, therefore it is less likely to affect the properties of the labelled receptors. Second, cell surface biotinylation can label the total amount of receptor at the cell surface and millions of cells are assayed at the same time, therefore it is more sensitive in identifying small differences in receptor endocytosis between treatment groups (Arancibia-Carcamo et al, 2006). Cell surface biotinylation has also been shown to be extremely useful in studying receptor endocytosis kinetics and turnover rate (Arancibia-Carcamo et al, 2006).

Other tools for studying receptor endocytosis

As we mentioned above, many effector molecules and distinct endocytic pathways are involved in receptor endocytosis and trafficking. Therefore genetic or pharmacological tools that inhibit or block specific steps in the endocytic pathways have been developed. Dominant negative dynamin mutant constructs and dynamin inhibitors are such tools that inhibit the ‘pinch off’ process of the coated vesicles from the membrane, thus inhibiting receptor endocytosis (Arancibia-Carcamo et al, 2006). Endosomal markers such as EEA1 have been used in differentiating various types of endosomes (Mu et al, 1995). Rab proteins such as Rab5 and Rab11 are also widely used as markers to identify specific sub-compartments of the endocytic pathway (Arancibia-Carcamo et al, 2006). Overall, these genetic or pharmacological tools are often used together with the antibody feeding or cell surface biotinylation methods to gain a better understanding of the mechanisms of receptor endocytosis (Arancibia-Carcamo et al, 2006).

1.5 Aims and objectives

As discussed, the eCB signalling has been postulated to play an important role in regulating neuroblast migration in the RMS in the postnatal brain; however, much of the supporting work was obtained using isolated neuroblasts or explant cultures. Furthermore, the relative importance of eCB signalling as compared with, for example, BDNF and FGFR signalling is not known. The aims of the first half of this thesis were to:-

- Use time-lapse live cell imaging of migratory neuroblasts to determine if DAGL-dependent eCB signalling regulates neuroblast motility and guidance in a regionally restricted manner within the relatively intact RMS.
- To compare the effects of inhibiting eCB, BDNF and FGFR signalling on neuroblast motility and guidance to gain insights into the relative importance of each pathway and to explore the possibility of direct-cross talk between them.

The regulated trafficking of neurotransmitter receptors in postsynaptic spines underpins short and long-term forms of synaptic plasticity. Some of these receptors (e.g. mGluR5) work “hand in glove” with DAGLα to regulate DSI and DSE. The aim of the second part of the thesis was to determine if DAGLα is similarly trafficked as this might serve as a mechanism contributing to eCB dependent synaptic plasticity. In this context the specific aims were to:-

- Develop assays to monitor DAGLα trafficking in a simple model cell type and determine which pathways regulating this.
- To determine if there are cell surface and intracellular pools of DAGLα in dendritic spines and if they are interchangeable.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Animals

P2-P3 mouse pups were bred from CD1 mice obtained from Charles River; all mouse pups in this thesis are from CD1 mice unless otherwise indicated. Fgfr1/2 $^{flox/flox}$ and Fgfr1/2 $^{flox/+}$ mice were bred and genotyped by Dr Kieran M. Jones (Dr M. Albert Basson group, Department of Craniofacial Development and Stem Cell Biology, King's College London). Embryonic day 18 Sprague Dawley rats (Harlan) were used for hippocampal neuron cultures. All procedures were performed in accordance with U.K. Home Office regulations (Animals Scientific Procedures Act, 1986).

2.1.2 General solutions

Phosphate Buffered Saline (PBS)

One PBS tablet (Oxoid) dissolved in a 100 ml of water and autoclaved: KCl (0.20 g/l),

KH2PO4 (0.2 g/l), NaCl (8 g/l) and Na2HPO4 (1.15 g/l).

Dissection media

Hank's Buffered Salt Solution (HBSS; Invitrogen) containing 5 mM HEPES (Sigma),

100 units/ml penicillin G and 100 µg/ml streptomycin (Invitrogen).

Brain slice collection media

Gey's Balanced Salt Solution (Invitrogen) supplemented with 0.45% glucose (Invitrogen).

Brain slice imaging media

Phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with

0.5% glucose, B27 supplement, 4 mM L-glutamine, 10 mM HEPES (pH 7.4), 100 units/ml penicillin G and 100 µg/ml streptomycin (Invitrogen) and 5% foetal calf serum (FCS).

Neuron seeding media

DMEM/F12 medium containing GlutaMax and 10% Horse serum (Gibco).

Neurobasal complete media

Neurobasal A medium (Life Technology) containing B27 supplement and GlutaMAX (Life Technology).

2.1.3 Constructs

pCX-EGFP

A kind gift from Dr. Masaru Okabe (Osaka University, Japan)

pCAG-Cre-IRES2-EGFP

From Addgene, plasmid 26646 (Woodhead et al, 2006)

HA-DAGLα

A HA peptide sequence (YPYDVPDYA) flanked by a glycine-serine-glycine-serine linker on either side was inserted into the first extracellular loop of Human DAGLα between H and E (performed by GenScript) in pcDNA™6.2-DEST/V5 construct cloned by gateway cloning by Dr Praveen K Singh. This construct was used to generate other DAGLα mutations.

HA-DAGLβ

A HA tag sequence (YPYDVPDYA) with GSGS as flanking on both side was inserted into the first extracellular loop of Human DAGLβ between D and C (performed by GenScript) in pcDNA™6.2-DEST/V5 construct cloned by gateway cloning by Dr Praveen K Singh. This construct was used to generate other DAGLβ mutations.

SEP-hCB1

A gift from Dr. Andrew J. Irving, University of Dundee (McDonald et al, 2007b)

Caveolin 1-GFP and GPI-GFP

Gifts from Dr. Oleg O. Glebov, King's College London (Glebov et al, 2006; Glebov & Nichols, 2004)

2.1.4 Drugs and factors

List of drugs used

Drug	Description	Concentration Range	Source
ACEA	CB1 receptor agonist	1 μM	Sigma
AM251	CB1 receptor antagonist	1 μM	Sigma
WIN 55, 212-2	CB1 receptor agonist	1 μM	Tocris Bioscience
JWH-133	CB2 receptor agonist	1 μM	Tocris Bioscience
JTE-907	CB2 receptor antagonist	1 μM	Tocris Bioscience
OMDM-188	DAGL inhibitor	1 μM	Kind gift from Dr. Di Marzo
PD173074	FGFR inhibitor	1 μM 1 mg/kg *in vivo*	Calbiochem
AZD4547	FGFR inhibitor	1 μM 1-12.5 mg/kg *in vivo*	Selleckchem
FGFR1-Fc	Recombinant Human FGF R1α (IIIc) Fc Chimera	1 μg/ml	R&D
TrkA-Fc	Recombinant human TrkA Fc Chimera	1 μg/ml	R&D
TrkB-Fc	Recombinant human TrkB Fc Chimera	1 μg/ml	R&D

2.1.5 Immunocytochemistry

Fixative

4% paraformaldehyde (PFA) in PBS, pH 7.4

Blocking/Permeabilising solution for cell lines

5% bovine serum albumin (BSA), 0.2% Triton-X 100 in 1 X PBS.

Blocking/Permeabilising solution for neurons

5% horse serum, 0.2% Triton-X 100 in 1 X PBS.

Primary/Secondary antibody solution for cell lines

5% bovine serum albumin (BSA), 0.2% Triton-X 100 in 1 X PBS.

Primary/Secondary antibody solution for neurons

5% horse serum, 0.2% Triton-X 100 in 1 X PBS.

Antibodies for immunocytochemistry and other fluorescent reagents

Antibody/Dye	Source	Species/ Conjugate	Dilution
βIII Tubulin	Abcam	Mouse monoclonal	1:1000
DCX	Abcam	Rabbit polyclonal	1:100
GFAP	Dako	Rabbit polyclonal	1:200
GFP	Invitrogen	Rabbit polyclonal	1:1000
RFP	Abcam ab62341	Rabbit polyclonal	1:500
DAGLα	Abcam ab81984	Goat	1:100
CB1 receptor	Gift from Dr Maurice Elphick	Rabbit	1:100
Flotillin 1	BD	Mouse	1:200
Cholera Toxin B subunit	Sigma C1655	N/A	1:500
Anti-HA	Roche 11867423001	Rat	1:100-1:200
Anti-V5	Abcam	Rabbit	1:1000
Rab 5	Cell signalling 3547	N/A	1:200
EEA 1	Abcam	N/A	1:500
Transferrin Receptor	Abcam ab9179	Mouse monoclonal	1:1000
LAMP 1	Abcam ab24107	Rabbit polyclonal	1:1000
Homer	Synaptic Systems	Rabbit	1:400
Bassoon	Abcam ab82958	mouse	1:1000
Transferrin 555	Life Technology	Alexa Fluor 555	1:1000
Hoechst	Sigma (B-2883)	N/A	1:10,000
Phalloidin A 488	Invitrogen	N/A	1:400
Goat Anti-Rat	Invitrogen	Alexa Fluor 488	1:1000
Goat Anti-Rat	Invitrogen	Alexa Fluor 594	1:1000
Goat Anti-Rat	Abcam	DyLight 650	1:500
Goat Anti-Mouse	Invitrogen	Alexa Fluor 488	1:1000
Goat Anti-Mouse	Invitrogen	Texas red	1:1000
Goat Anti-Mouse	Abcam	DyLight 650	1:500
Goat Anti-Rabbit	Invitrogen	Alexa Fluor 488	1:1000
Goat Anti-Rabbit	Invitrogen	Texas red	1:1000
Goat Anti-Rabbit	Abcam ab96886	DyLight 650	1:500

Mounting media

Fluorescent mounting media (Dako).

2.2 Methods

2.2.1 Cell culture

Cell lines

COS-7, HEK293, and Hela cells were cultured at 37°C and 5% CO_2 in DMEM media containing 10% FCS (Life Technology).

Primary RMS neuroblasts/explants

CD1 mice pups, postnatal day 5-7 (P5-P7), were sacrificed by cervical dislocation and then decapitated. Brains were collected in dissection medium. The most caudal third of the brain was removed and discarded. The remaining section of the brain was cut into 1.4 mm coronal slices using a Mcllwain Tissue Chopper. Slices were separated and the RMS was isolated from the tissue with the aid of a dissecting microscope. RMS explants were cut into fragments approximately 200-300 µm in diameter.

Dissociated RMS neuroblasts

To obtain dissociated neuroblasts, RMS fragments were triturated with 2 ml of HBSS containing 0.25% trypsin (Gibco) and 40 µl of DNAse I (1 mg/ml; Worthington), and left at 37°C for 2 minutes. The trypsin was inactivated with 5 ml of DMEM (Gibco) containing 10% FCS and the solution was centrifuged at 1,500 rpm for 5 minutes. After another two washes with DMEM + 10% FCS to remove any traces of trypsin, the pellet was re-suspended in pre-equilibrated (37°C/5% CO_2) Neurobasal complete medium (Gibco) containing B27 supplement, 2mM Lglutamine (Invitrogen), and 0.6% glucose (Sigma). Cells were plated onto 6 well plates (1,000,000 cells/well) or glass coverslips (30,000-50,000 cells/coverslip) coated with polyornithine (0.5 mg/ml; Sigma) and laminin (10 µg/ml; Sigma). Cells were maintained in Neurobasal complete medium at 37°C/5% CO_2 for 48-72 hours.

Hippocampal cultures

Dissociated hippocampal cultures were prepared from embryonic day 18 Sprague Dawley rats. Neurons were dissociated using 0.05% trypsin-EDTA (5 ml for 15 min at 37°C), followed by

washing and trituration and then plated onto 12 mm coverslips coated with poly-L-lysine (10 µg/ml) and laminin (10 µg/ml) in neuron seeding media in 4-well dishes. Media was changed to neurobasal complete media after neurons attached to the coverslips (~2h). Cultures were maintained at 37°C in a humidified incubator with 5% CO_2. Every 5-7 days, 10% culture media was added into the dish to maintain the culture.

2.2.2 Postnatal electroporation

P2 mice pups were anesthetized with isofluorane (0.6 L/min). Using a pulled glass capillary (O.D. 1.5 mm, I.D. 0.86 mm), 1-2 µl of 1 µg/µl pCX-EGFP plasmid (a kind gift from Dr. Masaru Okabe and Jun-ichi Miyazaki, Osaka University, Japan) or pCAG-IRES-GFP (for floxed mice experiments) was injected into the right ventricle. Animals were then subjected to five electrical pulses of 100 V for 50 ms with 850 ms intervals using the ECM 830 Square Wave Electroporation System (Harvard Apparatus, USA) and 5 mm tweezer electrodes coated with conductive gel (CEFAR, France). Animals were then reanimated under oxygen and returned to their mother.

2.2.3 Migration assays

Explant migration assay

RMS explants were embedded in growth factor-reduced phenol red free Matrigel (BD) on sterile glass coverslips and allowed to migrate for 6/24 hours in Neurobasal complete medium at 37°C/5% CO_2. Drugs were added to the Matrigel solution as well as the culture media and were present throughout the incubation period. Images of fixed and immunostained explants were captured on the Zeiss Apotome at 5X, 10X and 20X objectives. Distance migrated, the distance from the edge of the explant to the furthest migrated neuroblast perpendicular to the edge, was measured at 6 points around the periphery of the explant using ImageJ software. Approximately 15 to 20 explants were measured for each independent experiment. Explants in direct contact with other explants were excluded from the analysis.

For live imaging of explant migration, RMS explants were embedded in Matrigel in a 4 chamber 35mm Hi-Q4 culture dish (Nikon). Images were captured on the Nikon Biostation (an automatic multipoint time-lapse imaging system with controlled environment maintained at 37°C/5% CO_2) with 20X and 40X objectives every 3 minutes for 24 hours. Frames were played at a frequency of 10 frames per second. Tracking analysis was performed using Volocity software (Perkin Elmer), and limited to the first 9 hours of filming. Since neuroblasts from within the explant emerged at

different times during the filming period, cells were tracked for the first 4 hours of migration upon exiting the explant core. At least 40 cells were tracked for each condition in each experiment.

Migration in cultured brain slices

5-7 days post-electroporation, animals were sacrificed by cervical dislocation followed by decapitation. Brains were hemisected, and the right hemisphere (electroporated side) was mounted (midline face down) onto the Vibratome holder (Leica) using glue (Roti Coll). Brains were cut into 300 µm-thick sagittal sections in Gey's Balanced Salt Solution supplemented with 1% glucose (Invitrogen) using Vibratome (Leica), and slices containing the olfactory bulb were examined under a microscope for GFP positivity along the RMS. Slices with GFP signal were cultured on a Millicell insert (30 mm Organotypic PTFE 0.4 µm; Millipore) submerged in brain slice imaging media (with or without drugs) in a p35 glass bottom dish (MatTek) for 2 hours. The slices were then imaged at 37°C for 3 h using Perkin Elmer UltraView VoX spinning disk system equipped with an inverted Nikon Ti-E microscope using a Nikon CFI Super Plan Fluor ELWD 20X/0.45 objective and a 14 Hamamatsu Orca R2 camera. Frames were taken over a 100 to 150 µm interval at 2-4 µm z-steps every 3 min. Images were acquired using the Perkin Elmer Volocity acquisition software.

2.2.4 Transfection

For cell lines

Transfections of the cell lines with the constructs were carried out using the FuGene HD transfection reagent (Promega) in 6-well dish according to the FuGene HD protocol database. In brief, the cells were plated the day before transfection at a density of ~2 X 10^5 cells per well of a 6-well plate in 3 ml of complete cell medium. For the transfections, 3.3µg of DNA was added into OPTI-MEM media (Life Technology) to make total volume of 153µl, and then 12 µl of FuGene HD reagent was added. This was mixed carefully by vortexing briefly and incubating for 10-15 min at room temperature. Finally 150 µl of complex per well was added to the cells and mixed thoroughly. The cells were cultured overnight at 37°C and 5% CO_2. 24 hours after transfection, cells were generally trypsinised and seeded into 24 well plates for ICC/IF (described below) or in 6 well plates/10cm dishes for Western blotting analysis.

For hippocampal neurons

Hippocampal neurons were transfected using the NeuroMag Magnetofection system according to manufacturer's instruction. In brief, the indicated quantity of DNA was diluted in culture medium without serum and supplement (~1µg DNA in 50 µl medium). The DNA solution was added to the NeuroMag solutions by vigorous pipetting or brief vortexing and incubated at room temperature for 15 to 20 min. The NeuroMag/DNA complexes were added onto cells drop by drop and gently rocking the plate to ensure a uniform distribution. The cell culture plate was then placed on the magnetic plate for 15 min. Transgene expression was evaluated from 24 h to 7 days.

2.2.5 Receptor internalization and trafficking assay

Antibody feeding and receptor internalization assay in cell line

24 hours after transfection of HEK293/COS-7 cells with extracellular tagged constructs, an antibody against the extracellular epitope tag was added into the medium (DMEM supplemented with 25 mM HEPES and 0.5% BSA, pH 7.4) of live cells for 20 min at 4°C, the cells were then gently washed in PBS to remove unbound antibody and either fixed for a zero time point control or returned to the incubator for various times for internalization. Cells were then briefly fixed with 4% PFA and a fluorophore conjugated secondary antibody was used to label the antibodies that associate with the surface pool of the target protein. Cells were then permeabilized with 0.2% TritonX-100 and subsequently another fluorophore conjugated secondary antibody was used to label the extracellular tag associated with the internalized pool of the target protein. Images were taken by Zeiss LSM710 confocal microscopy by collecting random fields from various regions of the coverslip and analysed using ImageJ software.

Antibody feeding and receptor internalization assay in hippocampal neurons

Cultured hippocampal neurons were live labelled at room temperature instead of at 4°C due to the intolerance of neurons to low temperature and antibodies were incubated in neuron specific medium. Apart from these, the procedures for the assay are the same as for the cell lines.

Receptor recycling assay

COS-7 cells expressing HA-DAGLα were live labelled with rat anti-HA tag antibody (Roche) for 30 min at RT. The cells were either washed and fixed directly for a control or washed in a DMEM buffer (pH 3.0-3.5) before fixation to check acid wash efficiency. An anti-rat 488 secondary antibody was then used to detect the anti-HA antibody on the cell surface. Another group of COS-

7 cells were live labelled and PBS washed and returned back to the incubator for uptake for 30 min. After incubation, the cells were washed in acid buffer and either fixed (to test if the internalized antibody-enzyme complexes could still be detected after this acid wash step) or returned back to the incubator for another 30 min for the internalized antibody-enzyme complexes to recycle before fixation. An anti-rat 488 secondary antibody was used to detect the anti-HA antibodies that remained on the cell surface. After permeabilization, an anti-rat 594 secondary antibody was used to detect the anti-HA antibodies associated with the internalized HA-DAGLα. Images were taken by Zeiss LSM710 confocal microscopy by collecting random fields from various regions of the coverslip.

2.2.6 Immunohistochemistry

Gelatin-embedded sections

Electroporated brains were hemisected, fixed in 4% PFA overnight at 4°C, and then embedded in 8% gelatine, which was left to set overnight at 4°C. Gelatin-embedded brains were fixed in 4% PFA overnight at 4°C to harden the gelatin, and then cut on a Vibratome (Leica) into 70 µm-thick sagittal sections. Slices were blocked and permeabilised for 1 hour at room temperature in 5% BSA, 0.2% Triton-X, 0.01% sodium azide in PBS, and incubated with primary antibody overnight at 4°C in the same solution. Secondary antibodies were also diluted in the same solution and incubated for 2 hours at room temperature. Slices were washed in PBS, mounted onto glass slides using Dako mounting media, and covered with a 22x50 mm coverslip. Z-stack (every 1–2 µm) images of the stream were captured on a Zeiss LSM 710 confocal microscope using a 40X objective.

For the purpose of quantification, the RMS was classified into four anatomically distinct regions (Region A: descending arm of the RMS excluding the injection site; Region B: "elbow" of the RMS; Region C: region just after the elbow; D: RMS just before the OB) as previously described (Belvindrah et al, 2011). Process length was measured as the distance from the base of the cell body to the tip of the leading process using ImageJ software. Measurements were taken from all cells in each image. To determine orientation, all cells with their leading process outside a 180° angle relative to the position of the OB were considered to be disoriented.

2.2.7 Data analysis

Cell tracking of the time-lapse imaging

Volocity 6.0 software (Perkin Elmer) was used to track all the moving cells. Within each slice, 20-40 cells were tracked. Volocity software directly generates the value for the total distance (the sum of the distance between each time point) and the net distance (displacement: the distance between start and end point) and the value for the average speed for each cell travelled over 3 h. Only the cells that migrated a certain distance (total distance ≥ 100 μm) were considered as moving cells and included for statistical analysis (Nam et al, 2007). For cell migratory behaviour, the displacement/total distance ratio was calculated for each cell. According to this ratio, cells were classified as random (ratio: 0-0.4), intermediate (ratio: 0.4-0.6) or directed (ratio: 0.6-1) (Nam et al, 2007). For each treatment group the percentage of cells in each category was counted and the average calculated. For the speed of cell migration analysis, the average speed for each cell was calculated over a 3 h period by Volocity 6.0, the average of all the cells from each movie was then calculated, with this value averaged from all the movies for the same treatment condition and expressed with the SEM.

2.2.8 Statistical Analysis

Student's 2-tailed t-test and One-way or Two-way ANOVA followed by Bonferroni's post-tests were used for all statistical analysis. Where shown, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (all relative to control). For all graphs, each bar represents the mean ± SEM.

Chapter 3 Endocannabinoid signalling is required for directed cell migration within the RMS

3.1 Introduction

SVZ-derived neuroblasts can migrate to injured areas of the brain where they might limit damage and restore function (Arvidsson et al, 2002; Christie & Turnley, 2012; Young et al, 2011). Therefore understanding the molecular pathways regulating neuroblast migration is very important. Previous work has shown that the eCB system can regulate proliferation of neural stem cells in the SVZ (Gao et al, 2010; Goncalves et al, 2008) and the migration of their progeny neuroblasts out of RMS explants (Oudin et al, 2011a). It has been shown that the migrating neuroblasts in the RMS express components of the eCB system, including CB receptors, DAGLα, and MAGL (Oudin et al, 2011b). Detailed analysis of the eCB system using explant cultures of the RMS revealed that CB1 antagonists, CB2 antagonists, and DAGL inhibitors, were all capable of inhibiting neuroblast migration *in vitro* (Oudin et al, 2011a). Both CB1 and CB2 agonists (ACEA and JWH-133 respectively) significantly promoted the migration of RMS neuroblasts from the explants, and this effect was inhibited by their respective antagonists AM251 and JTE-907. Although combined addition of both CB1 and CB2 agonists enhanced migration out of explants, this effect was not greater than either one of the agonists on their own (Oudin et al, 2011a). Treatment of young mice with a single injection of CB1 or CB2 antagonists 24 hr before sacrifice decreases the length of the leading process of RMS neuroblasts *in vivo* (Oudin et al, 2011a). These results provided the first evidence that the eCB system is important for neuroblast migration in the RMS and also suggest that RMS neuroblast migration can be regulated by both CB1 and CB2 receptors (Oudin et al, 2011a).

However, the major body of the above work was on explant cultures where the polarity of the stream is lost thus making it difficult to determine if eCBs simply have a motogenic function or whether they also regulate guidance within or along the RMS. Also, the single *in vivo* experiment on neuroblast morphology examined in fixed brain slices was not able to provide dynamic information on neuroblast migration. A better understanding of migration dynamics can be obtained by real time imaging of neuroblasts within the relatively intact RMS in brain slice cultures (Nam et al, 2007). This can be used to identify factors that affect motility and local guidance, and

to determine if pathways operate in a regionally specific manner at the beginning or end of the stream.

The goal of the first part of this project was to test if eCB signalling regulates neuroblast migration in the relatively intact RMS by using time-lapse imaging in cultured brain slices. By doing so I also wanted to gain a better understanding of the dynamic role that the eCBs play in regulating local and longer-range guidance within the stream. In brief, *in vivo* electroporation was used to fluorescently label SVZ-derived neuroblasts in the post-natal mouse brain, acute brain slices were then prepared and cultured in the presence and absence of a wide array of pharmacological tools targeting the eCB system. Time-lapse imaging was then performed followed by analysis to study the neuroblast migration along the RMS (Sonego et al, 2013b). Our results show that the eCB signalling is required for both motility and guidance throughout the RMS.

3.2 Results

3.2.1 Cannabinoid antagonists perturb neuroblast migration in the RMS

In order to test the importance of the eCB system for neuroblast migration in the RMS, firstly we used a combination of CB1/2 antagonists (AM251 and JTE-907) in cultured brain slices as previous work has shown both CB1 and CB2 receptors play a role in neuroblast migration from RMS explant cultures (Oudin et al, 2011a). P2 mice pups were electroporated with pCX-EGFP to label stem cells in the ventral wall as described resulting in the presence of GFP-labelled neuroblasts within the stream 5-7 days later. Slices were equilibrated for 2 hours in control medium or medium containing a combination of CB1/2 antagonists (AM251 and JTE-907, both at 1 µM) before being imaged for 3 h. In our initial analysis we focused our attention on the descending arm of the RMS before the “elbow” region (region 1 in Figure 3-3). Individual neuroblasts were tracked and migration analysed. Representative images of migrating neuroblasts, tracked for 3 hours, for control and CB1/2 antagonist-treated slices are shown in Figure 3-1A,B. The white and red arrows highlight the position of two neuroblasts in each condition over the 3 hours period. Control neuroblasts migrated over longer distances towards the OB compared to neuroblasts in slices treated with the CB1/2 antagonists (Figure 3-1A,B). In addition, control cells displayed a predominant unipolar morphology usually oriented towards the OB, while cells treated with cannabinoid antagonists often displayed branched processes extending in all directions (Figure 3-1A,B, insets). The migration tracks of individual neuroblasts from representative slices are shown in Figure 3-1C-D). Under control conditions neuroblasts

tended to follow a similar path towards the OB (Figure 3-1C). Remarkably, when the CB receptors were inhibited, the cells could still move, but migration was clearly less directed (Figure 3-1D).

A detailed statistical analysis of cell migration dynamics in these experiments is summarised in Figure 3-2A-C. In control slices, neuroblasts were immobile (nucleus moved less than 2 μm) ~25% of their time, but this increased to almost 45% ($p<0.001$) when CB1/2 receptors were inhibited (Figure 3-2A). Interestingly when the cells did move in the presence of the CB1/2 antagonists they did so at a similar speed to control cells (60.5 ± 3.5 and 58.4 ± 3.4 μm/h, respectively). In agreement with a recent study (Bagley & Belluscio, 2010), when imaging over a 3 h period, ~70% of neuroblasts show net migration towards the OB in control medium (Figure 3-2B), and this was significantly reduced by treatment with the cannabinoid receptor antagonists to around 40% (Figure 3-2B). Total cell displacement was also substantially reduced (96.6 ± 7.9 μm for control and 69.6 ± 7.2 for treated) (Figure 3-2C). In summary, based on the measurement of several key indices of migration, the above results clearly support the hypothesis that the CB1/2 receptors regulate neuroblast migration in the intact RMS.

3.2.2 eCB signalling is required for directed cell migration within the RMS

Although the above results provide clear evidence of an important role for the eCB system in the regulation of neuroblast migration within the RMS, they do not address the important question as to whether this is important at both ends of the stream. To look for regional differences within the RMS, we analysed populations of neuroblasts sampled towards the beginning or end of the RMS as indexed by region 1 and region 2 in Figure 3-3A. Here we monitored the directionality of neuroblast migration by calculating the "meandering" index, i.e. the ratio between net displacement and total distance covered over the 3 h time period. According to this ratio, neuroblast movement can be classified as exploratory (ratio < 0.4), directed (ratio > 0.6), or intermediate (0.4 – 0.6) (Nam et al, 2007). In the RMS, close to the SVZ (Figure 3-3A), treatment with CB receptor antagonists resulted in a near two-fold increase in the number of exploratory neuroblasts at the expense of a near two-fold decrease in the number of cells migrating in a directed manner (Figure 3-3B). Very similar results were observed when neuroblasts were analysed in the RMS close to the OB (Figure 3-3C). Thus, we can conclude that eCB signalling is required for neuroblast motility and guidance within the RMS and that this pathway operates all along the RMS.

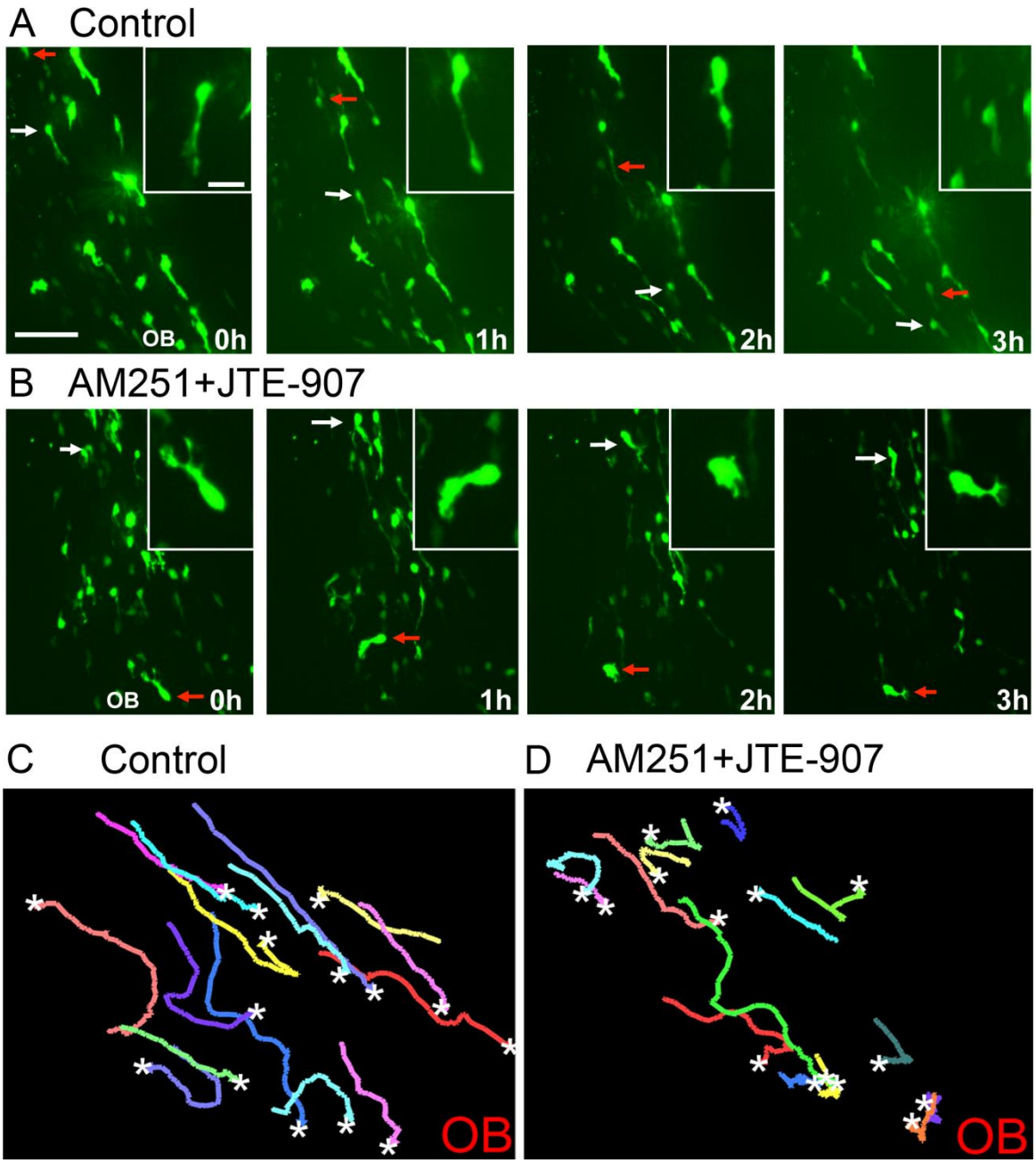

A Control
OB
0h
1h
2h
3h
B AM251+JTE-907
OB
0h
1h
2h
3h
C Control
D AM251+JTE-907
OB
OB

Figure 3-1 eCB antagonists perturb neuroblast migration in the RMS

Sagittal mouse brain slices with GFP-labeled neuroblasts were prepared 5-7 days after *in vivo* postnatal electroporation of P2 mice with pCX-EGFP. Slices were cultured with vehicle or drugs (as indicated) for 2 hours and subsequently imaged for 3 hours in the same medium. Time-lapse movies were made from the descending arm of the RMS in slices treated with drugs targeting the eCB system (the CB1/2 antagonists AM251+JTE-907, both at 1 µM) were analyzed using Volocity. Representative pictures of slices treated with vehicle (A) or the CB1/2 antagonists AM251+JTE-907 (B) are shown. Arrows follow two neuroblasts in each frame. Insets show magnifications of the neuroblast indicated by the white arrows (A) or red arrow (B). Cells migrate towards OB with time in control slice while AM251+JTE-907 treated cells show less directed movements. Representative migratory tracks of 15 cells over 3 hours from a control (C) or CB1/2 antagonist-treated brain slice (D). White stars mark the tracking end point of each cell. The OB label shows the location of the olfactory bulb in each brain slice. Bars, 35 µm for (A–B), 10 µm for insets.

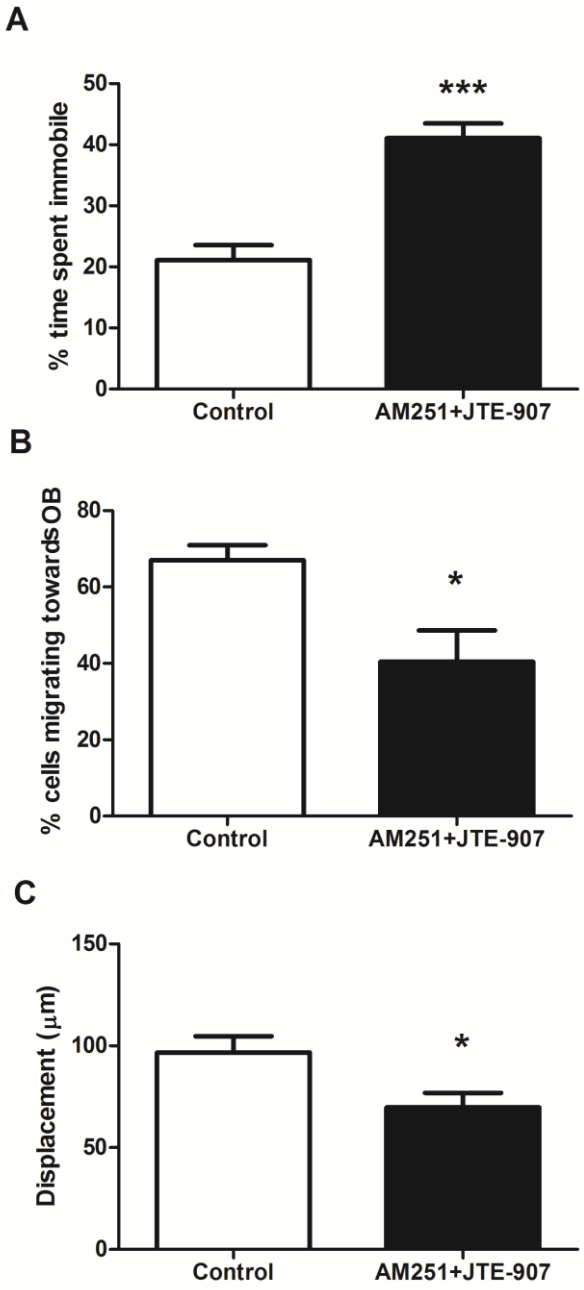


Figure 3-2 eCB antagonists perturb neuroblast migration in the RMS

Quantification of cell migration from control slices or slices treated with the CB1/2 antagonists AM251+JTE-907 (all at 1 µM). Time-lapse movies were all made from the descending arm of the RMS. Cells treated with the CB1/CB2 antagonists spent more time immobile (A). Incubation with CB antagonists also significantly decreased the percentage of neuroblasts migrating towards the OB (B) and the overall cell displacement of the cells (C). Graphs show mean ± s.e.m. (n=7 brain slices for each condition, ~ 15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

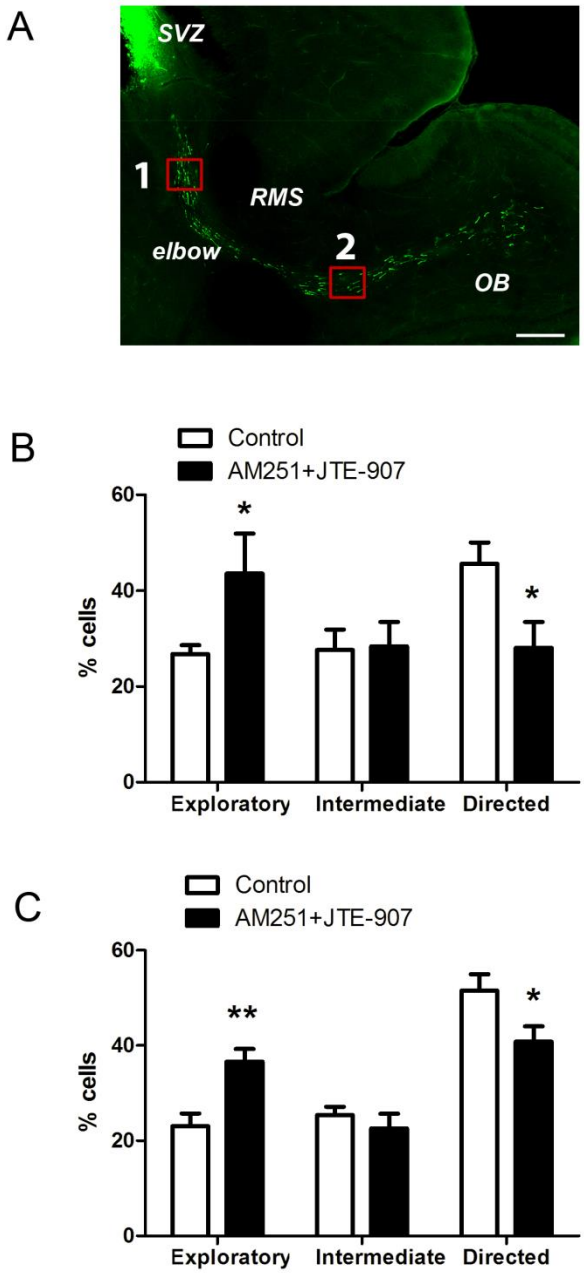


Figure 3-3 Inhibiting eCB signalling perturbs neuroblast migration at both ends of the RMS

Five to seven days after *in vivo* electroporation of pCX-EGFP in P2 mice, brain slices were cultured with vehicle or drugs as indicated for 2 h followed by time-lapse imaging for 3 h in the same medium. (A) A typical sagittal brain slice showing labelled neuroblasts. The two red rectangular boxes labelled 1 and 2 indicate the imaging sites in the beginning (caudal) and end (rostral) of the RMS respectively. Incubation with CB1 and CB2 antagonists (AM251 + JTE-907, both at 1 μM) significantly increased the percentage of exploratory neuroblasts at the expense of directed neuroblasts in both regions of the RMS (B, caudal RMS; C, rostral RMS). Graphs show mean ± s.e.m. (n= 6 brain slices for each condition, ~15–30 cells analysed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Bar, 400 μm.

3.2.3 DAGL inhibitor perturb neuroblast migration in the RMS

The above results provide clear evidence for eCB signalling regulating migration, but they do not address the nature of the eCB driving the response. As discussed previously, there is considerable evidence for 2-AG being the principle eCB for the central nervous system, and DAGLs (especially DAGLα) have been identified as the major enzymes for 2-AG synthesis in the brain (Bisogno et al, 2003; Gao et al, 2010). Therefore, to test if DAGL activity is required for neuroblast migration in the RMS, a DAGL inhibitor, OMDM-188 (Ortar et al, 2008) was added to the cultured brain slices. As before, P2 mice pups were electroporated with pCX-EGFP and slice cultures containing GFP-labelled neuroblasts within the stream were established 5-7 days later. Slices were equilibrated for 2 h in control medium or medium containing OMDM-188 (1 μM) before being imaged for 3 h. Again, our initial analysis was focused on the descending arm of the RMS before the "elbow" region (region 1 in Figure 3-3A). Individual neuroblasts were then tracked and migration analysed. The migration tracks of individual neuroblasts from control and DAGL inhibitor-treated slices are shown in Figure 3-4A,B. Control neuroblasts migrated over longer distances towards the OB compared to neuroblasts in slices treated with the DAGL inhibitor (Figure 3-4A,B). Under control conditions neuroblasts tended to follow a similar path towards the OB (Figure 3-4A). Remarkably, when DAGL was inhibited, the cells could still move, but migration was clearly less directed and the cells tends to have shorter migration tracks (Figure 3-4B). A detailed statistical analysis of cell migration dynamics in these experiments is summarised in Figure 3-4C-E. In control slices, neuroblasts were immobile (nucleus moved less than 2 μm) ~25% of their time, similar to CB receptor antagonists treated slices, this increased to almost 50% ($p<0.001$) when the DAGL enzymes were inhibited (Figure 3-4C). In control medium, ~70% of neuroblasts show net migration towards the OB (Figure 3-4D), and this was significantly reduced by treatment with the DAGL inhibitor to around 50% (Figure 3-4D). Total cell displacement was also substantially reduced (Figure 3-4E).

3.2.4 DAGL signalling is required for directed cell migration within the RMS

To look for regional differences within the RMS, we analysed populations of neuroblasts sampled towards the beginning or end of the RMS as indexed by region 1 and region 2 in Figure 3-3A. Again, we monitored the directionality of neuroblast migration by calculating the "meandering" index, i.e. the ratio between net displacement and total distance covered over the 3 h time period. Similar results were obtained in both regions of the stream when DAGL activity was inhibited

compare to the CB receptor antagonists' treatment (Figure 3-5). More specifically, close to the SVZ in the RMS (Figure 3-5A, region 1), treatment with the DAGL inhibitor resulted in a near two-fold increase in the number of exploratory neuroblasts at the expense of a near two-fold decrease in the number of cells migrating in a directed manner (Figure 3-5B). A significant effect was also observed when neuroblasts were analysed in the RMS close to the OB (Figure 3-5C), treatment with the DAGL inhibitor resulted in a near three-fold increase in the number of exploratory neuroblasts at the expense of a near two-fold decrease in the number of cells migrating in a directed manner (Figure 3-5C). Thus, we can conclude that DAGL-dependent eCB signalling is required for neuroblast motility and guidance within the RMS and that this pathway operates all along the RMS.

3.2.5 eCB agonists increase cell motility without disrupting directionality in the RMS

In the above experiments we have tested the effects of inhibiting eCB signalling on neuroblast migration within the RMS. There are also some excellent small molecule agonists for the CB1 and CB2 receptors, and although it is perhaps not clear what they might do to migration in brain slices, it was nonetheless of interest to test this. The CB receptor agonists have been shown to increase neuroblast motility and promote cell migration out the explant of the RMS *in vitro* (Oudin et al, 2011a). To test whether the CB receptor agonists simply have a motogenic function or whether they also regulate guidance along the RMS, we used a combination of CB1/2 agonists (ACEA and JWH-133) in cultured brain slices. P2 mice pups were electroporated with pCX-EGFP and slice cultures containing GFP-labelled neuroblasts within the stream were established 5-7 days later. Slices were equilibrated for 2 h in control medium or medium containing ACEA and JWH-133 (both at 1 µM) before being imaged for 3 h. Our initial analysis was focused on the descending arm of the RMS before the "elbow" region. Individual neuroblasts were then tracked and migration analysed. Quantification of cell migration from control slices or slices treated with the CB1/2 agonists ACEA+JWH-133 is shown in Figure 3-6. Cells treated with the CB1/2 agonists migrate at a higher speed (67.02 ± 2.9 µm/h) compared to control cells (57.11 ± 1.9 µm/h) suggesting that the receptors are not maximally active under control conditions (Figure 3-6A). Interestingly, incubation with CB agonists does not significantly alter the percentage of neuroblasts migrating towards the OB (Figure 3-6B). We then analysed the populations of neuroblasts sampled towards the beginning or end of the RMS to look at the guidance effect of CB agonists along the RMS, again no significant difference were found between treated and

control brain slices (Figure 3-6C, pooled from beginning and end of the RMS). Thus we conclude that the eCB agonists increase the cell motility, albeit by a relatively small amount. However, perhaps the key observation from this section is that the agonists do not have any significant effect on guidance within the stream. This result will be discussed in detail below, but it provides substantive evidence against the notion that long range eCB gradients might operate along the RMS as addition of exogenous agonists would be expected to “flatten” any such gradient.

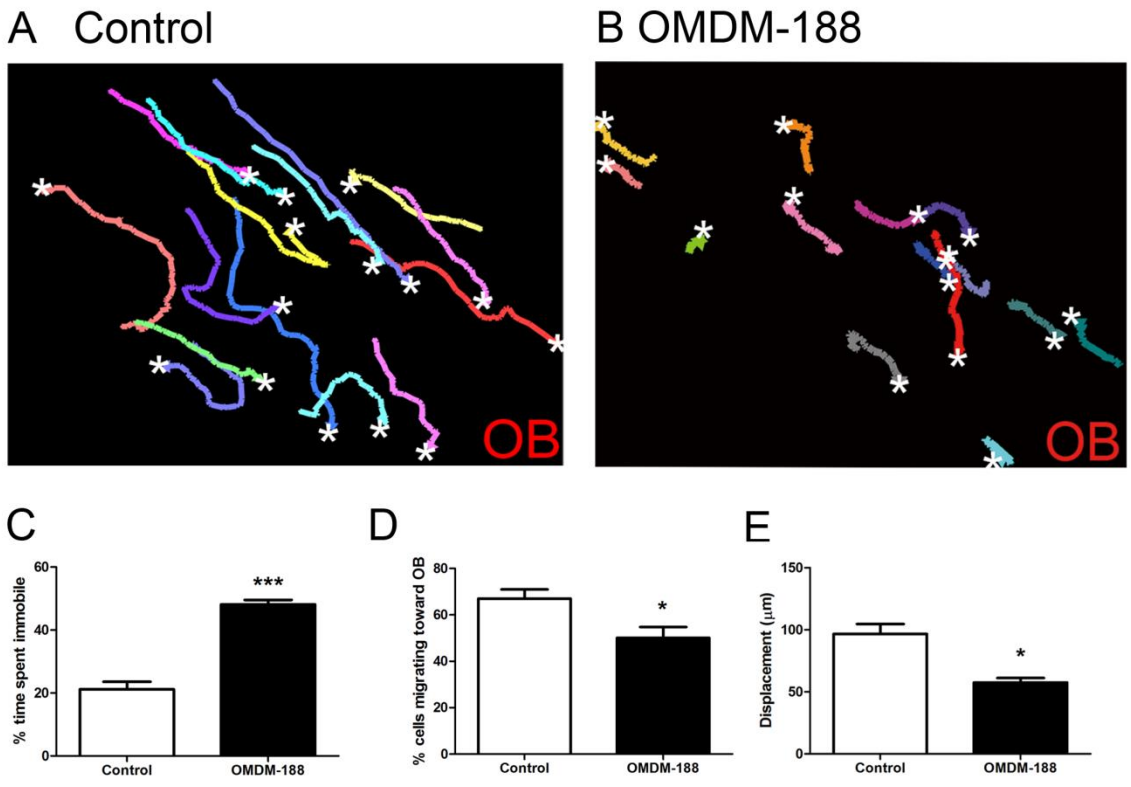


Figure 3-4 A DAGL inhibitor perturbs neuroblast migration in the RMS

Time-lapse movies made from the descending arm of the RMS in slices treated with a DAGL inhibitor (OMDM-188) were analyzed using Volocity. Representative migratory tracks of 15 cells over 3 hours from a control (A) or DAGL inhibitor-treated (OMDM-188 at 1 μM) brain slice (B). White stars mark the tracking end point of each cell. The OB label shows the location of the olfactory bulb in each brain slice. Cells migrate towards OB with time in control slice while OMDM188 treated cells show less directed movements. (C-E) shows quantification of cell migration from control slicesor slices treated with OMDM-188 at 1 μM. Cells treated with the DAGL inhibitor spent more time immobile (C). Incubation with the DAGL inhibitor also significantly decreased the percentage of neuroblasts migrating towards the OB (D) and the overall cell displacement (E). Graphs show mean ± s.e.m. (n=7 brain slices for each condition, ~ 15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

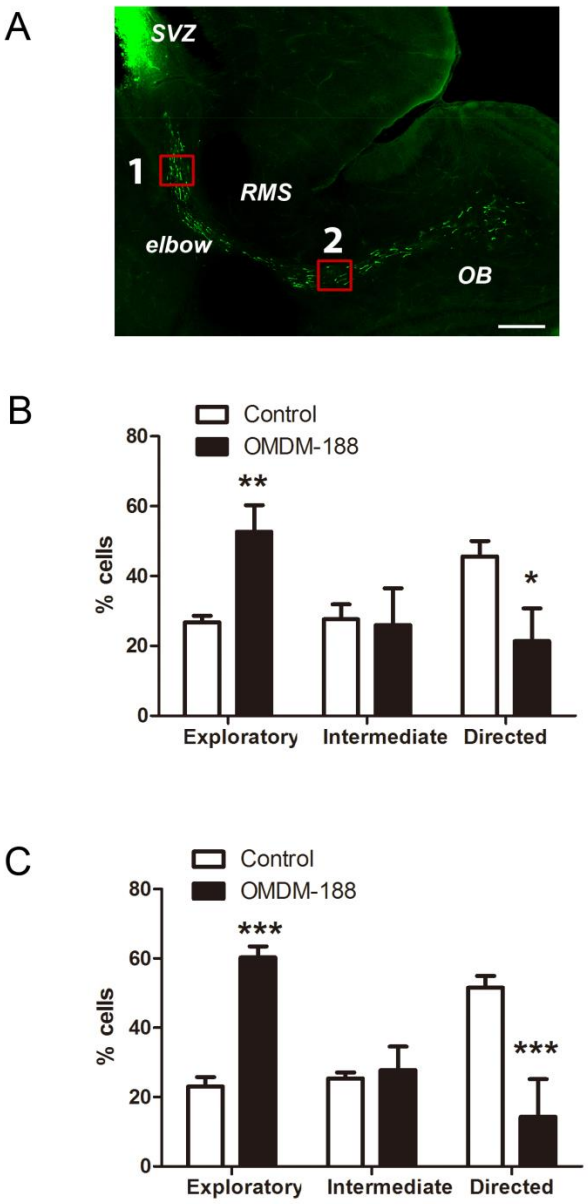


Figure 3-5 Inhibiting DAGL perturbs neuroblast directionality along the RMS

Five to seven days after *in vivo* electroporation of pCX-EGFP in P2 mice, brain slices were cultured with vehicle or drugs for 2 h followed by time-lapse imaging for 3 h in the same medium. (A) A typical sagittal brain slice showing labelled neuroblasts. The two red rectangular boxes labelled 1 and 2 indicate the imaging sites in the beginning (caudal) and end (rostral) of RMS, respectively. Incubation with a DAGL inhibitor (OMDM-188, 1 μM) significantly increased the percentage of exploratory neuroblasts at the expense of directed neuroblasts in both regions of the RMS (B, caudal RMS; C, rostral RMS). Graphs show mean ± s.e.m. (n= 6 brain slices for control and 4 for treated, ~15 – 30 cells analysed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Bar, 400 μm.

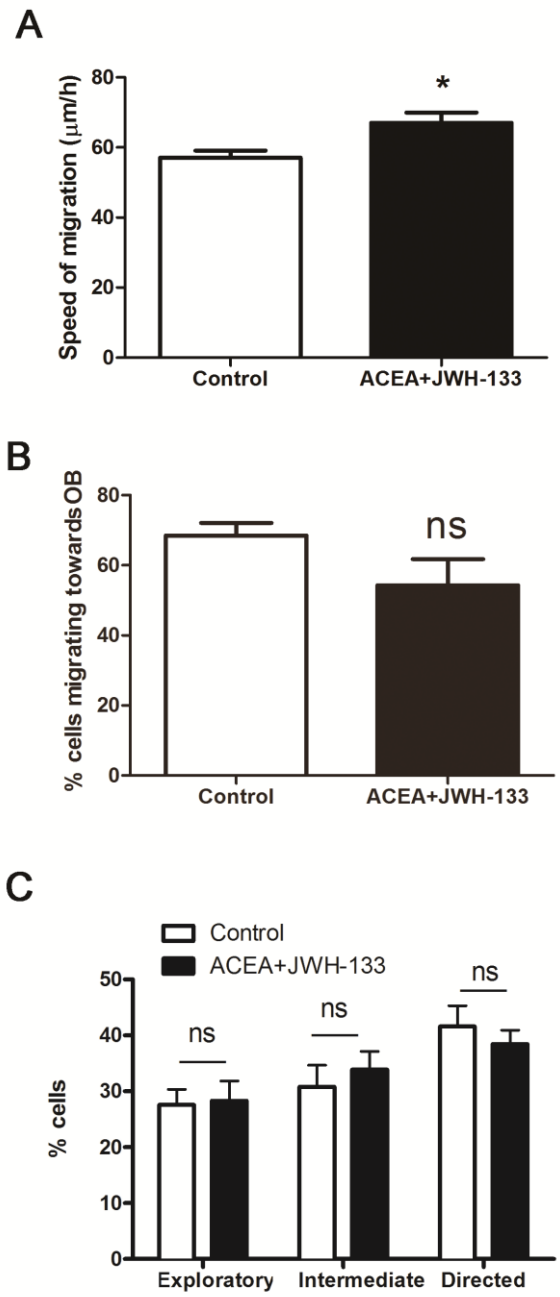


Figure 3-6 The effects of eCB agonists on neuroblast migration in the RMS

Quantification of cell migration from control slices or slices treated with the CB1/2 agonists ACEA+JWH-133 (both at 1 μM). Time-lapse movies were all made from the descending arm of the RMS. Cells treated with the CB1/2 agonists migrate at a higher speed compared to control cells (A). Incubation with CB agonists does not significantly decrease the percentage of neuroblasts migrating towards the OB (B) and does not significantly disrupt the directionality (C). Graphs show mean ± s.e.m. (n=7 brain slices for each condition, ~ 15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Chapter 4 FGFR signalling is required for directed cell migration at the beginning of RMS

4.1 Introduction

In the last chapter, we used time-lapse imaging to characterise the role of the eCB system in the regulation of neuroblast migration and concluded that the eCB system is required for neuroblast motility and guidance within the RMS and that this pathway operates all along the RMS. Although we know the eCB system plays a role in the RMS, we don't know its' relative importance compared to other signalling pathways that also operate in the RMS. Indeed, most studies tend to focus on a single signalling system at a time, and examples include BDNF, GABA, integrin, and slits (Bagley & Belluscio, 2010; Bolteus & Bordey, 2004; Chiaramello et al, 2007; Mobley & McCarty, 2011; Nguyen-Ba-Charvet et al, 2004; Snapyan et al, 2009). Therefore, despite the fact that numerous molecules have been found to be important for neuroblast migration in the RMS, how they relate to each other and integrate their signalling pathways is far from fully understood (Ming & Song, 2011). The eCB pathway is well suited to directly cross-talk with receptor tyrosine kinases and other receptors that stimulate the synthesis of DAG, the DAGL-substrate. Insights into possible interactions can be gleaned by directly comparing the relative importance of the eCB signalling to that of other putative guidance cues on the migration of the neuroblast in the RMS. In this context we have treated brain slices with tools to perturb fibroblast growth factor receptor (FGFR) signalling and TrkB signalling as both can cross-talk to the eCB pathway (discussed below). In this chapter, we report on FGFR signalling system while the TrkB results will be discussed in the next chapter.

Previous studies in our lab have provided evidence for direct cross-talk between the eCB system and FGFRs (Williams et al, 2003). Evidence was obtained that supports the hypothesis that activation of FGFR signalling can lead to activation of PLCγ to synthesize DAG, with DAGL then using this substrate to generate 2-AG to activate the CB1 receptors. This direct cross-talk between the FGFR and the CB1 receptor promotes axonal growth, a biological response that shares many features with cell migration (Williams et al, 2003). It is also possible that activation of FGFR signalling might lead to direct cross-talk with eCB signalling via the same PLCγ dependent mechanism for neuroblast migration in the RMS as has been reported for axonal growth.

The FGF/FGFR signalling superfamily comprises four closely related receptors (FGFR1-4), and numerous growth factor ligands. The amino acid sequences of the FGFRs are highly conserved between different members of the family and throughout evolution (Burke et al, 1998). Studies in the past two decades have clearly shown that FGFs and their receptors play key roles in multiple biological processes including tissue repair, haematopoiesis, angiogenesis, embryonic development and neural proliferation (Reuss & von Bohlen und Halbach, 2003). At least 18 FGFs have been found to date, although not all the FGF subtypes can be detected in the central nervous system or in all species. The important role of FGFs on cognitive process and neural proliferation in the adult brain has been demonstrated (Reuss & von Bohlen und Halbach, 2003). More pertinent to this study, it has been shown that in the SVZ, the proliferating precursor cells express mRNA for both FGFR1 and FGFR2, and may respond to FGF-2 released by a non-proliferating cell type present in the SVZ (Frinchi et al, 2008). Another study using immunostaining for FGF-2 revealed a caudal-rostral expression gradient along the RMS, with stronger labelling in the SVZ region than the OB. Analysis of transcript levels by qPCR revealed higher levels of FGFR1 and 2 than FGFR3 in the postnatal SVZ, with very low expression of FGFR4 detected (Garcia-Gonzalez et al, 2010). In addition, the FGFR1 has been shown to be expressed by SVZ derived neuroblasts in culture, and treatment of SVZ derived explant cultures with FGF-2 significantly increased migration from the explants, and this effect was suggested to be mediated by the FGFR1(Garcia-Gonzalez et al, 2010).

The hypothesis that a caudal-rostral FGF-2 gradient along the SVZ and RMS drives the migration of neuroblasts by working on FGFR1 and its downstream pathways has not been critically tested *in vivo*. In this study, postnatal electroporation and real-time imaging methods are applied to test if FGFR signalling regulates neuroblast motility and guidance in the RMS slice culture model, and whether this pathway operates in a regional specific manner. Administration of an FGFR inhibitor to living animals was also used to test the effect of FGFR signalling on neuroblast migration in vivo. I have also characterised neuroblast migration in the RMS in mice that have a conditional deletion of FGFR1/2 to compare the consequence of chronic loss of the receptors with acute loss of receptor function. Our results show that directly targeting FGFR signalling with drugs perturbs neuroblast migration in the RMS. This was seen following treatments with agents that inhibit activity of the receptor, agents that compromise the function of conventional receptor ligands, and also by application of excess amounts of exogenous FGF-2 to the slice culture that might be

expected to “flatten” the putative gradient of FGF-2 reported by others. In all instances the migration of the neuroblasts becomes more random rather than directed. Interestingly, this effect on migratory neuroblasts is only seen at the beginning of the RMS, with the same treatments having no effect on cells within the end of the RMS. These results support the hypothesis the caudal-rostral FGF-2 gradient along the SVZ and RMS drives the migration of neuroblasts by working on FGFR to activate its downstream signalling pathways. Surprisingly, studies with the FGFR1/2 conditional knockout animals did not support the hypothesis, but this might be accounted for by established redundancy in the MAPK pathway downstream of the FGFR in the neural stem cell lineage (Sutterlin et al, 2013) and this is discussed in detail below.

4.2 Results

4.2.1 FGFR inhibitors perturb neuroblast migration in the RMS

To test the importance of FGFR activity for neuroblast migration in the RMS, we first used two highly selective FGFR inhibitors, AZD4547 and PD173074 that inhibit receptor activity by competing for ATP binding to the activated receptor (Gavine et al, 2012; Mohammadi et al, 1998). P2 mice pups were electroporated with pCX-EGFP to label stem cells in the ventral wall resulting in the presence of GFP-labelled neuroblasts within the stream 5-7 days later. Brain slice cultures were equilibrated for 2 h in control medium or medium containing AZD4547 or PD173074 (both at 1 μM) before being imaged for 3 h. Again, we initially focused our attention on the descending arm of the RMS before the “elbow” region (region 1 in Figure 4-2). Individual neuroblasts were tracked and migration analysed. Visual inspection of neuroblast migration tracks showed a very clear reduction in displacement and a loss of guidance when the FGFR inhibitors were present. The migration tracks of individual neuroblasts from representative slices are shown in Figure 4-1A-C. Under control conditions neuroblasts tended to follow a similar path towards the OB (Figure 4-1A), while the cells in the slices treated with FGFR inhibitors clearly migrate in a less directed manner (Figure 4-1B-C).

A detailed statistical analysis of cell migration dynamics in these experiments is summarised in Figure 4-1D-F. In control slices, neuroblasts were immobile (nucleus moved less than 2 μm) ~32% of their time, but this increased to ~40% ($p<0.05$) and ~50% ($p<0.001$) in the presence of AZD4547 and PD173074 respectively (Figure 4-1D). In control medium, ~75% of neuroblasts show net migration towards the OB (Figure 4-1E), and this was significantly reduced by treatment with the AZD4547 to around 50% (Figure 4-1E). Moreover, total cell displacement was also

substantially reduced (113.8 ± 7.1 µm for control, 80.2 ± 6.6µm and 73.9 ± 9.4 µm for AZD4547 and PD173074 treated) (Figure 4-1F). In summary, based on the measurement above, the results clearly support the hypothesis that FGFR signalling regulates neuroblast migration in the intact RMS.

4.2.2 FGFR inhibitors perturb directed cell migration at beginning but not end of the RMS

Next we determined whether FGFR is important at both ends of the stream by analysing populations of neuroblasts sampled towards the beginning or end of the RMS as indexed by region 1 and region 2 in Figure 4-2A. Again we monitored the directionality of neuroblast migration by calculating the "meandering" index, i.e. the ratio between net displacement and total distance covered over the 3 h time period. Our results showed that close to the SVZ in the RMS (Figure 4-2A), treatment with both FGFR inhibitors resulted in a significant reduction in the number of neuroblasts showing directed migration with a corresponding increase of exploratory neuroblasts and neuroblasts in the intermediate group. Remarkably, this effect was only seen with neuroblasts sampled at the beginning of the RMS, with no significant effects seen on any of the measured parameters with neuroblasts sampled at the end of RMS (Figure 4-2C). Thus, it would appear that FGFR signalling is required for neuroblast motility and guidance in the RMS, but unlike the eCB pathway this pathway is only operative at the beginning of the stream.

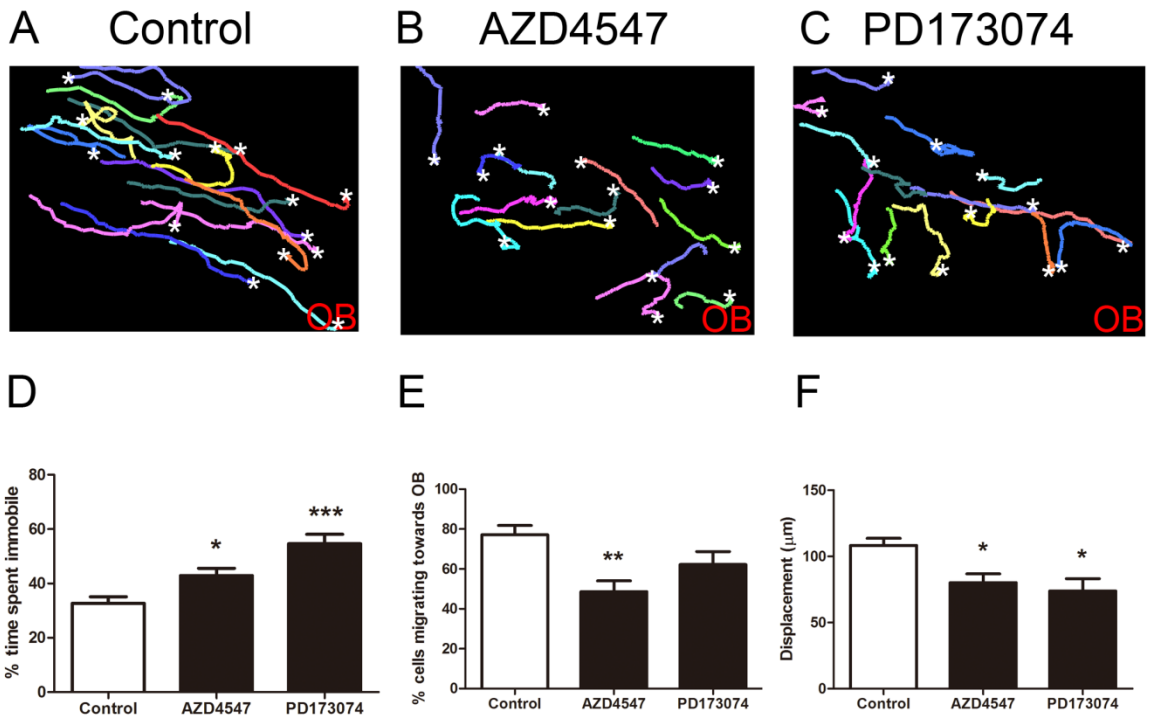


Figure 4-1 FGFR inhibitors perturb neuroblast migration in the RMS

Time-lapse movies made from the descending arm of the RMS in control slices and slices treated with FGFR inhibitors (AZD4547 and PD173074) were analyzed using Volocity. Representative migratory tracks of 15 cells over 3 hours from a control (A) or AZD4547 (B) or PD173074-treated (both at 1 μM) brain slice (C). White stars mark the tracking end point of each cell. The OB label shows the location of the olfactory bulb in each brain slice. Cells migrate towards the OB with time in control slices while inhibiting FGFR signalling results in less directed cell movements. (D-F) shows quantification of cell migration from control slices or slices treated with AZD4547 or PD173074. Cells treated with the FGFR inhibitors significantly increase the percentage of time spend immobile (D). Incubation with AZD4547 or PD173074 also significantly decreased the percentage of neuroblasts migrating towards the OB (E) and the overall cell displacement (F). Graphs show mean ± s.e.m. (n=7 brain slices for each condition, ~ 15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

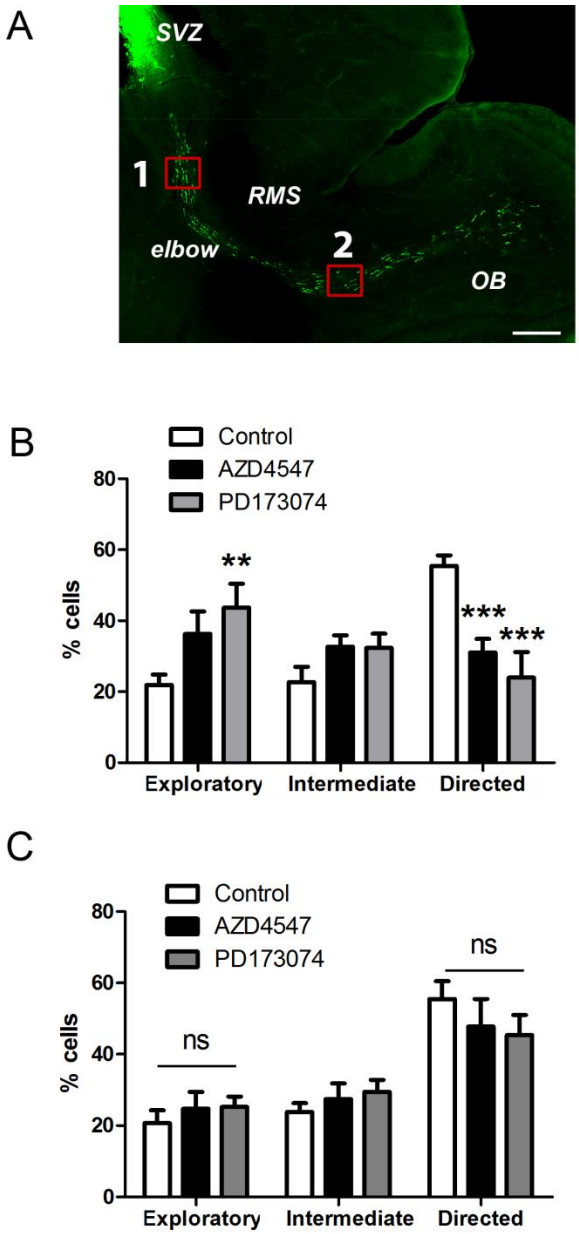


Figure 4-2 FGFR inhibitors perturb neuroblast migration at the beginning but not the end of the RMS

Five days after *in vivo* electroporation of pCX-EGFP in P2 mice, brain slices were cultured with vehicle or AZD4547 or PD173074 (both at 1 µM) for 2 h followed by time-lapse imaging for 3 h in the same medium. Neuroblast migration was filmed and analysed in the beginning and end of RMS (regions 1 and 2 in A). Incubation with AZD4547 or PD173074 significantly increased the percentage of exploratory neuroblasts at the expense of directed neuroblasts towards the beginning of the RMS (B), while the same treatments do not have any significant effect at the end of the RMS compared to control (C). Graphs show mean ± s.e.m. (n=6-8 brain slices for each condition, ~15-30 cells analysed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Bar, 400 µm

4.2.3 A FGFR1-Fc perturb neuroblast migration in the RMS

The results above clearly support the role of FGFR activity regulating migration at the beginning of the RMS, but give no insights into the nature of the ligand(s) that might activate the FGFR(s). Previous studies have shown that the FGFRs can be directly activated by the “conventional” FGF ligands; however a more recent body of work has established that a number of cell adhesion molecules can act as “surrogate” ligands and also activate FGFRs independently of the conventional ligands (Boscher & Mege, 2008; Cavallaro et al, 2001; Doherty & Walsh, 1996; Saffell et al, 1997; Sanchez-Heras et al, 2006; Turner & Grose, 2010; Williams et al, 1994a; Williams et al, 2001). The recombinant human FGFR1 Fc chimera (FGFR1-Fc) is a chimeric molecule consisting of the Fc region of human IgG1 fused to the extracellular domains of FGFR1 alpha (IIIc) (Martinez-Torrecuadrada et al, 2005). It will bind FGFR1 ligands like FGF-2 and prevent them from interacting with the cell expressed receptors. Therefore we used a FGFR1-Fc chimera to trap any FGFR1 ligands present within the RMS. As before, P2 mice pups were electroporated with pCX-EGFP and slice cultures containing GFP-labelled neuroblasts within the stream were established 5-7 days later. Slices were equilibrated for 2 h in control medium or medium containing FGFR1-Fc (1 μg/ml) before being imaged for 3 h. Our initial analysis was focused on the descending arm of the RMS before the “elbow” region (region 1 in Figure 4-2). Individual neuroblasts were then tracked and migration analysed. The migration tracks of individual neuroblasts from control and FGFR1-Fc-treated slices are shown in Figure 4-3A,B. Control neuroblasts migrated over longer distances towards the OB compared to neuroblasts in slices treated with the FGFR1-Fc (Figure 4-3A,B). Under control conditions neuroblasts tended to follow a similar path towards the OB (Figure 4-3A). When the FGFR1-Fc was added to the brain slices, the cells moved in less directed manner and they tended to have shorter migration tracks (Figure 4-3B). A detailed statistical analysis of cell migration dynamics in these experiments is summarised in Figure 4-3C-E. In control slices, neuroblasts were immobile (nucleus moved less than 2 μm) ~28% of their time, this increased to almost 45% ($p<0.05$) when treated with FGFR1-Fc (Figure 4-3C). In control medium, ~75% of neuroblasts show net migration towards the OB (Figure 4-3D), and this was significantly reduced by treatment with the FGFR1-Fc to ~60% (Figure 4-3D). Total cell displacement was also significantly reduced (Figure 4-3E).

4.2.4 FGFR1-Fc perturb directed cell migration within the RMS

Next we wanted to test for regional differences of the FGFR1-Fc effect within the RMS. Populations of neuroblasts sampled towards the beginning or end of the RMS were analysed as indexed by region 1 and region 2 in Figure 4-2A. Again, we monitored the directionality of neuroblast migration by calculating the “meandering” index. The results showed that the FGFR1-Fc treatment had a significant effect towards the beginning of the RMS, but no significant effect has been seen in the end of the RMS. More specifically, close to the SVZ in the RMS (Figure 4-4A, region 1), treatment with FGFR1-Fc resulted in a significant decrease in the number of cells migrating in a directed manner with a trend of increasing the number of exploratory neuroblasts (Figure 4-4B). No significant effect was observed when neuroblasts were analysed in the RMS close to the OB (Figure 4-4C), treatment with FGFR1-Fc even resulted in a small trend to decrease the number of cells migrating in an exploratory manner at the expense of increasing the number of directed neuroblasts (Figure 4-4C). Thus, we can conclude that FGFR ligand(s) drive neuroblast migration and guidance at the beginning but not the end of the RMS. At first sight this might point to the conventional FGFR1 ligands driving the response, but the ability of the soluble FGFR1-Fc to inhibit CAM stimulated neurite outgrowth has not been determined.

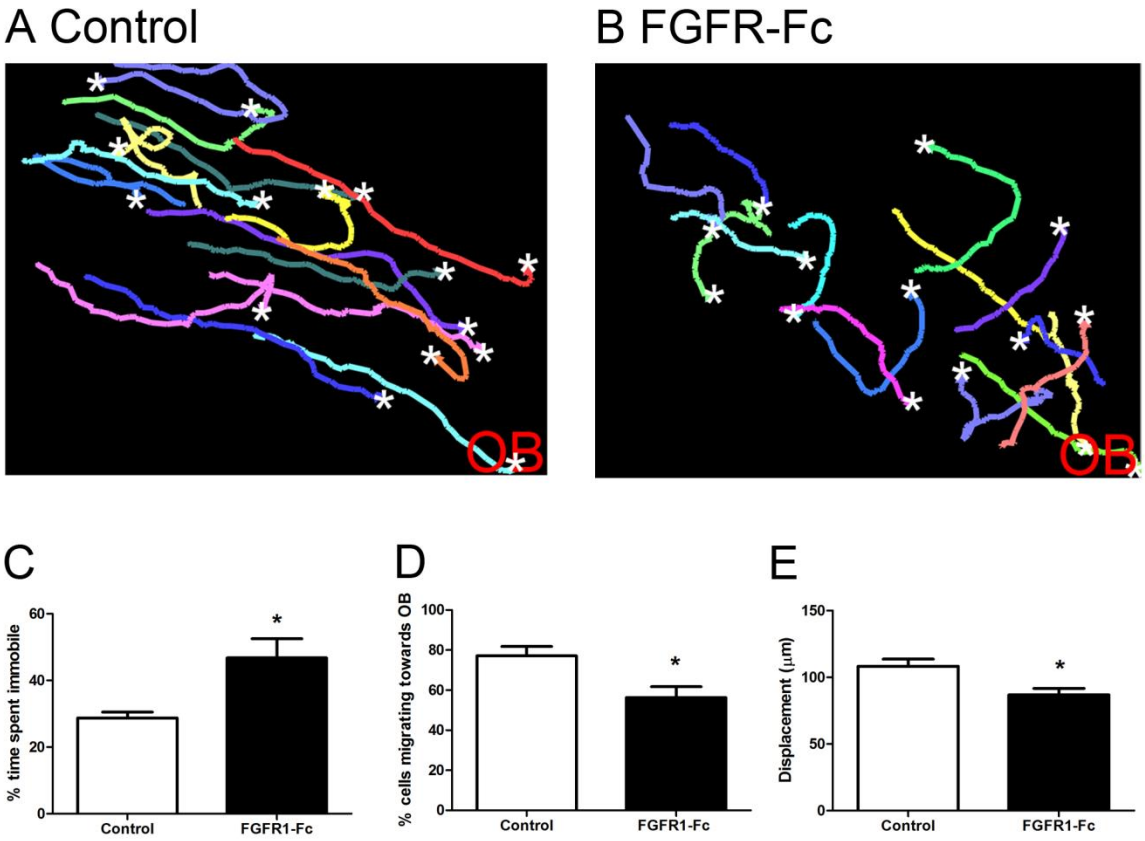


Figure 4-3 An FGFR1-Fc perturbs neuroblast migration in the RMS

Time-lapse movies made from the descending arm of the RMS in slices treated with an FGFR1-Fc and were analyzed using Volocity. Representative migratory tracks of 15 cells over 3 hours from a control (A) or FGFR1-Fc-treated (1 μg/ml) brain slice (B). White stars mark the tracking end point of each cell. The OB label shows the location of the olfactory bulb in each brain slice. Cells migrate towards the OB with time in control slices, while cells treated with the FGFR1-Fc show less directed movements. (C-E) shows quantification of cell migration from control slices or slices treated with FGFR1-Fc. Cells with treated with the FGFR1-Fc significantly increase the percentage of time spend immobile (C). Incubation with FGFR1-Fc also significantly decreased the percentage of neuroblasts migrating towards the OB (D) and the overall cell displacement (E). Graphs show mean ± s.e.m. (n=7 brain slices for each condition, ~ 15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

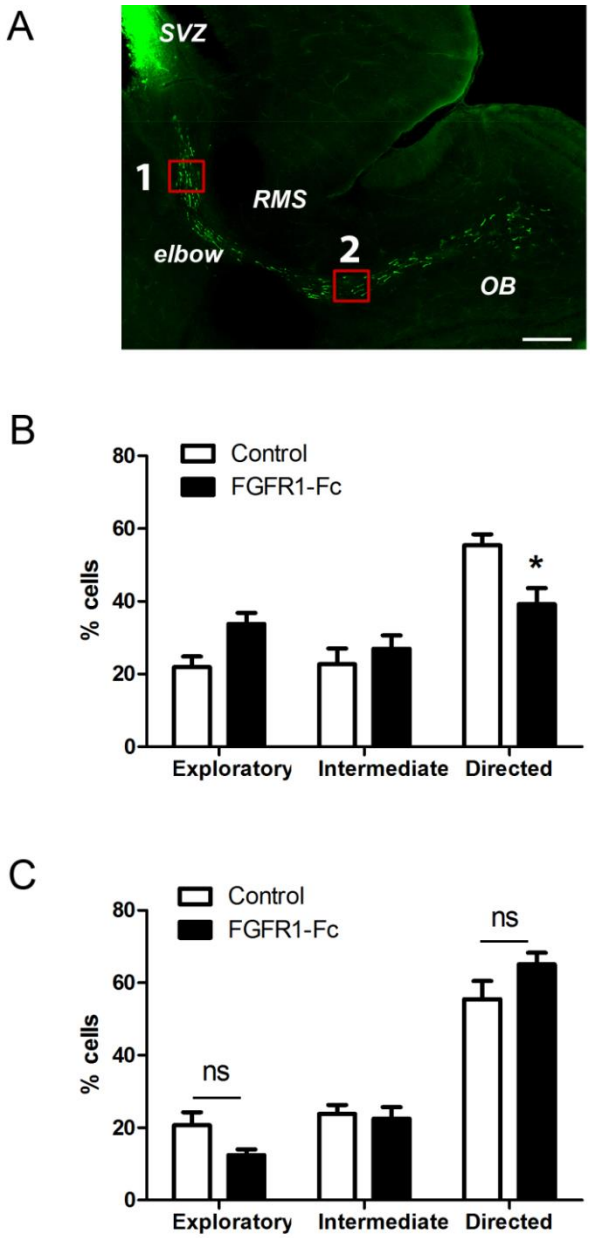


Figure 4-4 An FGFR1-Fc perturbs neuroblast directionality at the beginning but not the end of the RMS

Five days after *in vivo* electroporation of pCX-EGFP in P2 mice, brain slices were cultured with vehicle or an FGFR1-Fc (1 µg/ml) for 2 h followed by time-lapse imaging for 3 h in the same medium. Neuroblast migration was filmed and analysed in the beginning and end of RMS (regions 1 and 2 in A). Incubation with FGFR1-Fc significantly increased the percentage of exploratory neuroblasts at the expense of directed neuroblasts towards the beginning of the RMS (B), while the same treatment does not have significant effect compared to control slices and end of the RMS (C). Graphs show mean ± s.e.m. (n=6-8 brain slices for each condition, ~15-30 cells analysed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Bar, 400 µm

4.2.5 Disruption of FGF-2 signalling in the RMS perturb neuroblast migration at the beginning but not end of the RMS

The FGFR1-Fc would be expected to inhibit the function of FGF-2 and it has been reported that both FGF-2 and FGFR1 are expressed in the SVZ as well as in the OB and FGF-2 can promote migration from the explant *in vitro* (Garcia-Gonzalez et al, 2010; Hebert et al, 2003; Mudo et al, 2007). Moreover, a caudual-rostral gradient of FGF-2 has been reported within the RMS (Garcia-Gonzalez et al, 2010). Therefore, it's very likely that FGF-2 signalling through FGFR1 is responsible for the gradient effect we reported above. To test whether FGF-2 is important for neuroblast migration, we first inhibited FGF-2 in the RMS by using a specific FGF-2 blocking antibody bFM-1 (Matsuzaki et al, 1989). Also we wanted to test whether a gradient of endogenous FGF-2 might be playing an instructive role in migration as suggested by others (Garcia-Gonzalez et al, 2010). Therefore, we treated the slice cultures with a relatively high concentration of exogenous FGF-2 (50 ng/ml) to "flatten" any FGF-2 gradient. P2 mice pups were used for electroporation, and slice cultures were prepared 5-7 days after electroporation. Slices were equilibrated for 2 h in control medium or medium containing bFM-1 (10 µg/ml) or FGF-2 (50 ng/ml) before being imaged for 3 h. Populations of neuroblasts sampled towards the beginning or end of the RMS were analysed as indexed by region 1 and region 2 in Figure 4-5A. Again, we monitored the directionality of neuroblast migration by calculating the "meandering" index over the 3 h time period. Visual inspection of neuroblast migration tracks showed a very clear reduction in displacement and a loss of guidance when the FGF-2 neutralizing antibody was present or when high concentration of exogenous FGF-2 was added, and again these effects were only seen at the beginning of the RMS. Detailed analysis on the meandering index is shown in Figure 4-5B,C. Both treatments resulted in a significant decrease in the number of cells migrating in a directed manner with a trend seen of increasing the number of cells in the "exploratory" and "intermediate" groups at the beginning of the RMS (Figure 4-5B). In contrast, we again failed to detect a significant effect of the treatments on the migratory parameters in neuroblasts sampled at the end of the stream (Figure 4-5C). Thus, based on the result with the blocking antibody we can propose that FGF-2 / FGFR signalling is important for the directed migration of the neuroblasts in the RMS, but in contrast to eCB signalling, this pathway only operates towards the beginning of the RMS. The results from the exogenous FGF-2 suggested that the "correct" concentration of FGF-2 is important in the RMS; these results support the hypothesis that endogenous FGF-2 might form a gradient in the RMS to guide neuroblast migration.

4.2.6 Targeting FGFR signalling affects cell displacement at the beginning but not end of the RMS

In order to substantiate the above results we also analyzed the cell displacements (the distance between begin and end point of a migrating cell) at both the beginning and end of the RMS for all the treatments that target the FGF-2/FGFR pathway. In this context we compared the effects of the two FGFR inhibitors, the FGFR1-Fc, the FGF-2 neutralizing antibody and treatment with an “excess” of exogenous FGF-2 on the cell displacement at both ends of the RMS (Figure 4-6). Consistent with the results shown above, all treatments significantly decrease the cell displacement at the beginning of the RMS, but none affected displacement towards the end of the RMS (Figure 4-6). These results, together with those reported above, provide very substantial support for an important role for FGFR signalling in the regulation of directed neuroblast migration at the beginning of the RMS, and suggest that this is driven by FGF-2 acting on the FGFR1 in the migratory neuroblasts. Furthermore, they show that this pathway operates in a regionally restricted manner, functioning at the beginning but not the end of the stream.

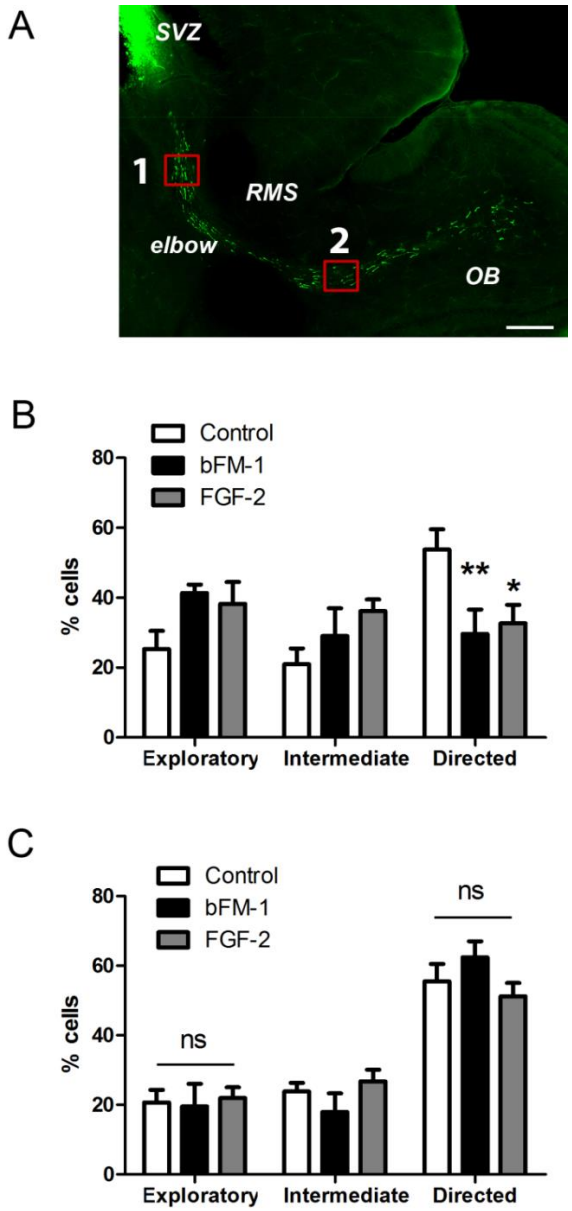


Figure 4-5 Targeting FGF-2 signalling affects neuroblast migration in the beginning but not the end of the RMS

Acute mouse brain slices containing GFP-labelled neuroblasts were prepared 5 – 7 days after *in vivo* electroporation of pCX-EGFP, cultured with vehicle or drugs for 2 h and imaged for 3 h in the same medium. FGF-2 signalling was targeted using an FGF-2 neutralizing antibody (bFM-1at 10 µg/ml), or by treatment with FGF-2 (at 50 ng/ml). Both treatments decreased the percentage of cells migrating in a directed fashion and increased the amount of exploratory neuroblasts in the beginning (A) but not in the end of RMS relative to the vehicle control (B). Graphs show mean ± s.e.m. (n= 5 – 8 brain slices for each condition, ~15 – 30 cells analysed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

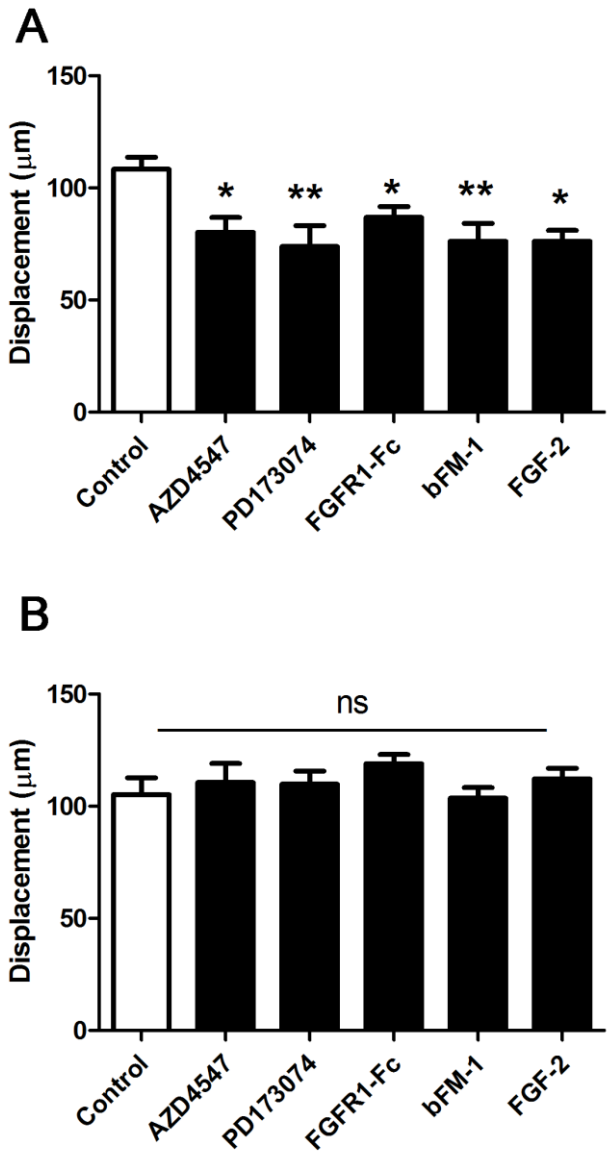


Figure 4-6 Targeting FGFR signalling affects cell displacement at the beginning but not end of the RMS

Quantification of total cell displacement the neuroblast migrated over 3 h period at the beginning (A) and end of the RMS (B) (regions 1 and 2 in Figure 4-5A). FGFR signalling was targeted using AZD4547, PD173074 (both at 1 μM) or an FGFR1-Fc (1 μg/ml) in brain slice cultures. A FGF-2 neutralizing antibody bFM-1 (10 μg/ml) or FGF-2 (50 ng/ml) were also added to the slice cultures followed by time-lapse imaging. All treatments significantly decrease the overall cell displacement at the beginning of the RMS relative to vehicle treated slices (A) while the cell displacement at the end of the RMS is not significantly disrupted by any of the treatments (B). Graphs show mean ± s.e.m. (n=5-7 brain slices for each condition, ~ 15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.2.7 Regional effects of an FGFR inhibitor on neuroblast morphology *in vivo*

We have shown that inhibiting FGFR signalling can disrupt neuroblast migration in the acute brain slice cultures. To further test the role of FGFR signalling in the RMS, we also treated P7 mice (same source and species of mice as used above for slice cultures) by I.P. administration of the FGFR inhibitor AZD4547 to determine if there is any evidence that the neuroblast migration can be comprimised by inhibiting the FGFR *in vivo*. The mouse pups were electroporated with pCX-EGFP at P2 and 5 days later treated with the FGFR inhibitor AZD4547 (12.5 mg/kg I.P., two doses with a 12 hour interval). After 24 h, brains were removed, fixed, sliced and immunostained for GFP. Representative pictures of neuroblasts in animals treated with vehicle (Figure 4-7A) and AZD4547 (Figure 4-7B) in the descending arm of RMS are shown in Figure 4-7. Visual inspection of the neuroblasts in the RMS treated with AZD4547 suggested a decrease in the length of the leading process (Figure 4-7B). Morphometric analysis of this parameter measured on neuroblasts sampled throughout the entire RMS revealed that there was a significant decrease of the length of the leading process (Figure 4-7C). Detailed analysis of neuroblasts sampled in different regions of the RMS (see cartoon in Figure 4-8) showed that treatment with AZD4547 significantly reduced the length of the leading process towards the beginning and not the end of the RMS (Figure 4-8). These results provides evidence for FGFR signalling regulating neuroblast morphology *in vivo*, and supports the conclusion made with slice cultures pointing to regional differences in FGFR signalling within the RMS.

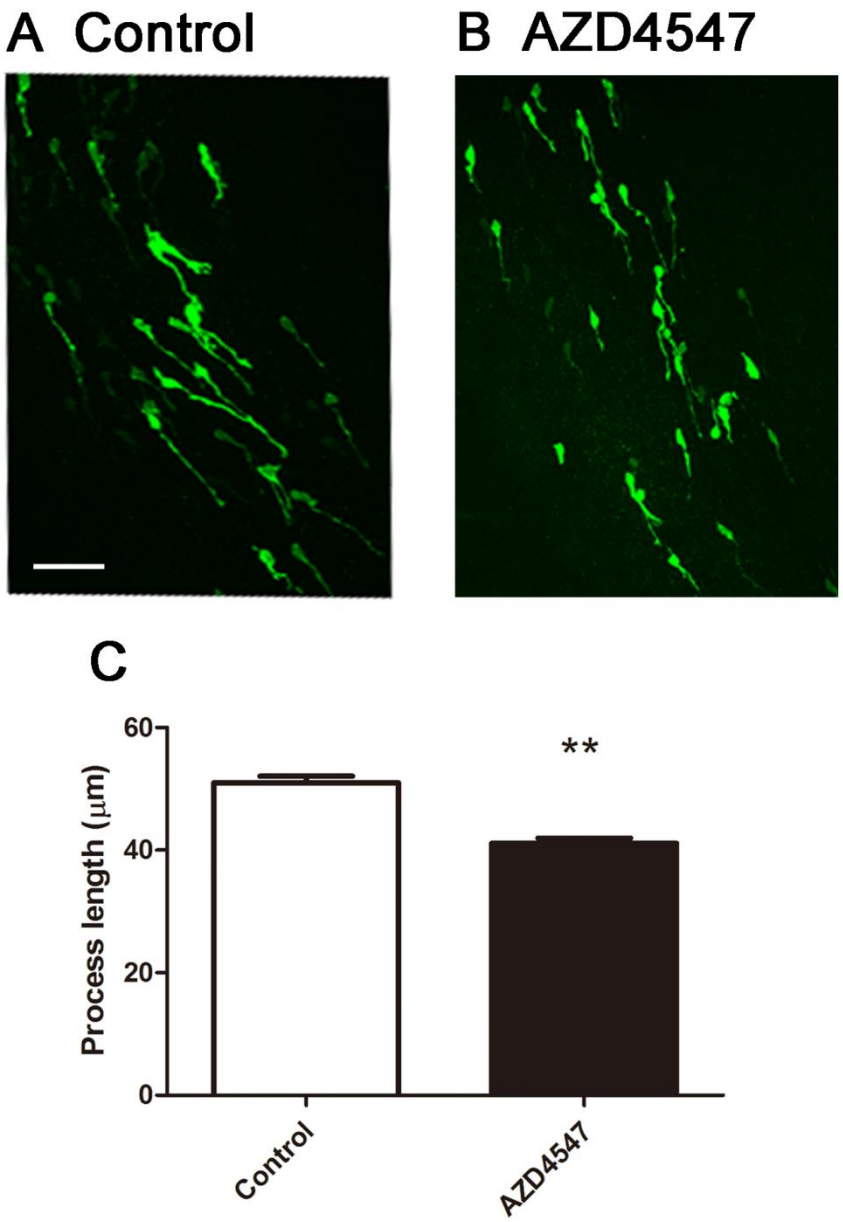


Figure 4-7 An FGFR inhibitor alters the morphology of neuroblasts within the RMS *in vivo*

P2 mouse pups were electroporated with pCX-EGFP and 5 days later treated with the FGFR inhibitor AZD4547 (12.5 mg/kg I.P., two doses with a 12 hour interval). After 24 h, brains were fixed, sliced and stained for GFP. Representative pictures of migrating neuroblasts in animals treated with vehicle (A) and AZD4547 (B) in the descending arm of RMS are shown. Treatment with the FGFR inhibitor significantly decreased the process length of migrating neuroblasts in the RMS (C, data pooled from the whole RMS). Graphs show mean ± s.e.m. (n= 4 animals for control and 3 for drug treated, 6 consecutive slices were analysed per brain, ~100 cells analysed per brain); *p< 0.05, **p<0.01. Bar, 50 μm for (A-B).

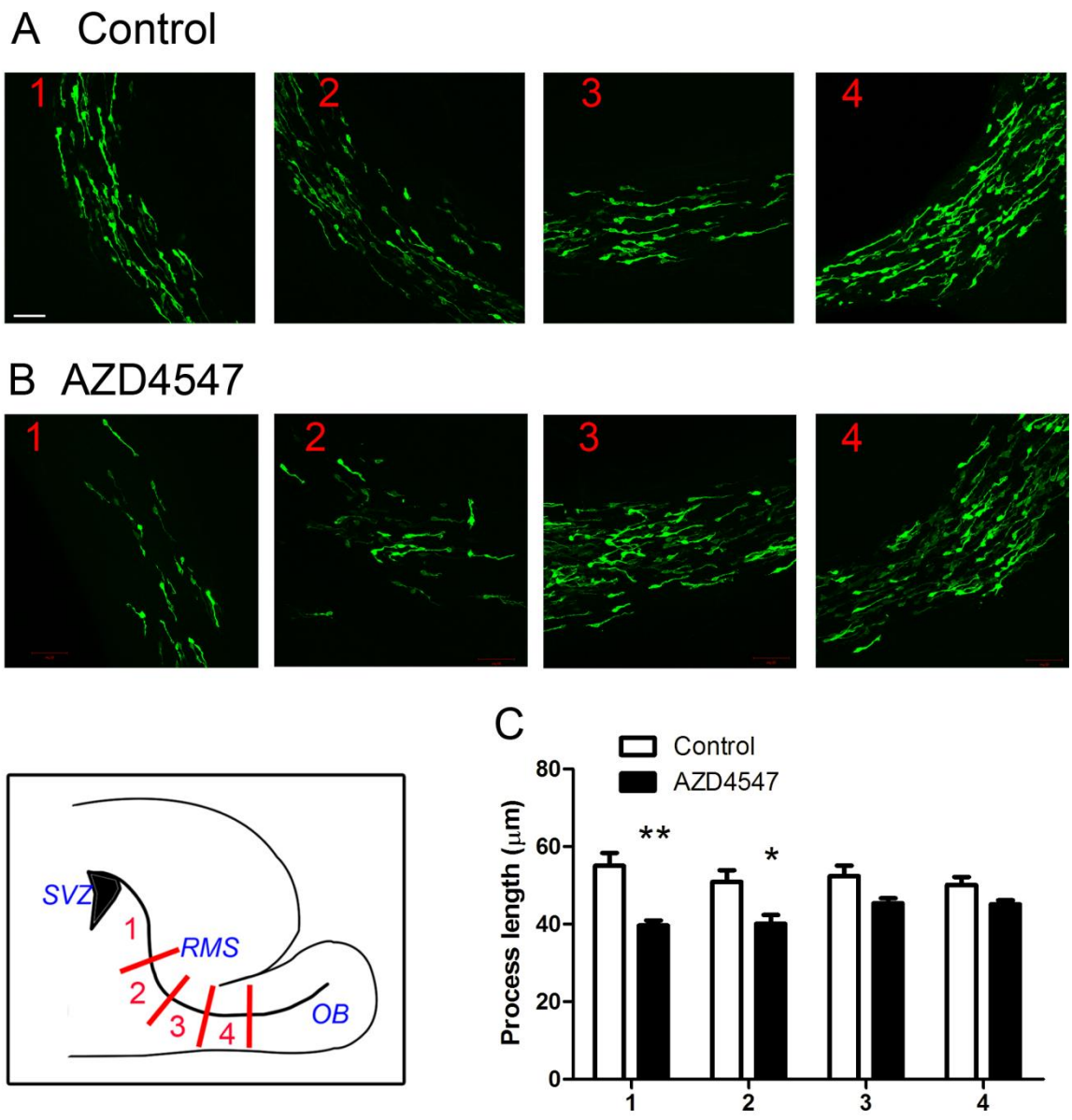


Figure 4-8 Regional effects of the FGFR inhibitor on neuroblast morphology in the RMS *in vivo*

P2 mouse pups were electroporated with pCX-EGFP and 5 days later treated with the FGFR inhibitor AZD4547 (12.5 mg/kg I.P., two doses with a 12 hour interval). After 24 h, brains were fixed, sliced and stained for GFP. Representative pictures of migrating neuroblasts in animals treated with vehicle (A) and AZD4547 (B) in 4 different regions along the RMS (labelled 1-4 as depicted in the cartoon). Treatment with the FGFR inhibitor significantly decreased the process length of migrating neuroblasts only in regions 1 and 2 of the RMS (C). Graphs show mean ± s.e.m., (n= 4 animals for control and 3 for drug treated, 6 consecutive slices were analysed per brain, ~100 – 200 cells analysed per region); *p< 0.05, **p<0.01. Bar, 50 μm for (A-B).

4.2.8 FGF-2 and FGFR1 transcript expression in the SVZ and OB of the postnatal mice

The above results clearly support a role for FGF-2 and its receptor FGFR1 in the RMS. Although a previous study has suggested that a caudal-rostral gradient of FGF-2 exists in the RMS by using an FGF-2 antibody (Garcia-Gonzalez et al, 2010), we have not been able to reproduce this finding using some of the available anti-FGF-2 antibodies (results not shown). We therefore turned to the quantification of the level of receptor and ligand transcripts to see if we could find a basis for the existence of the putative gradient. To address this, we dissected tissue from the SVZ and OB of P7 mice pups (Figure 4-9) followed by mRNA extraction and RT-qPCR quantification. The level of FGFR1 and FGF-2 transcripts were normalized to the internal control GAPDH (Figure 4-9B). Transcripts encoding FGF-2 and FGFR1 were detectable both in SVZ and OB. However, the expression levels of FGF-2 were ~20% lower in the OB compared with SVZ, while the expression level of FGFR1 showed no significant difference (Figure 4-9). Thus results showed here might provide the basis for a caudal-rostral FGF-2 gradient in the RMS; however a very accurate determination of ligand levels along the RMS would be required to substantiate this as it is not clear if a 20% reduction in transcript levels would provide a clear basis for establishment of a ligand gradient.

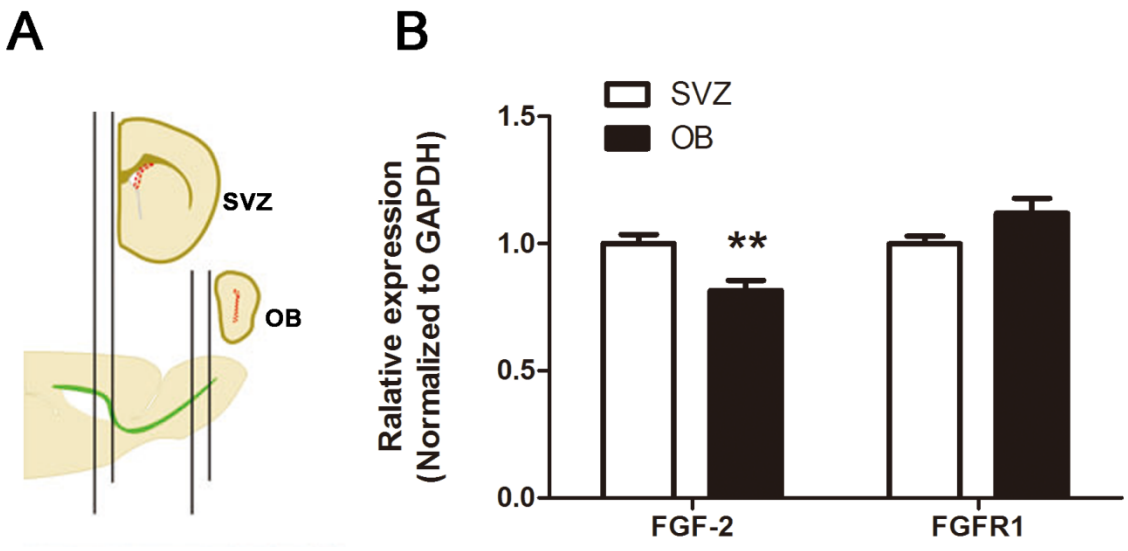
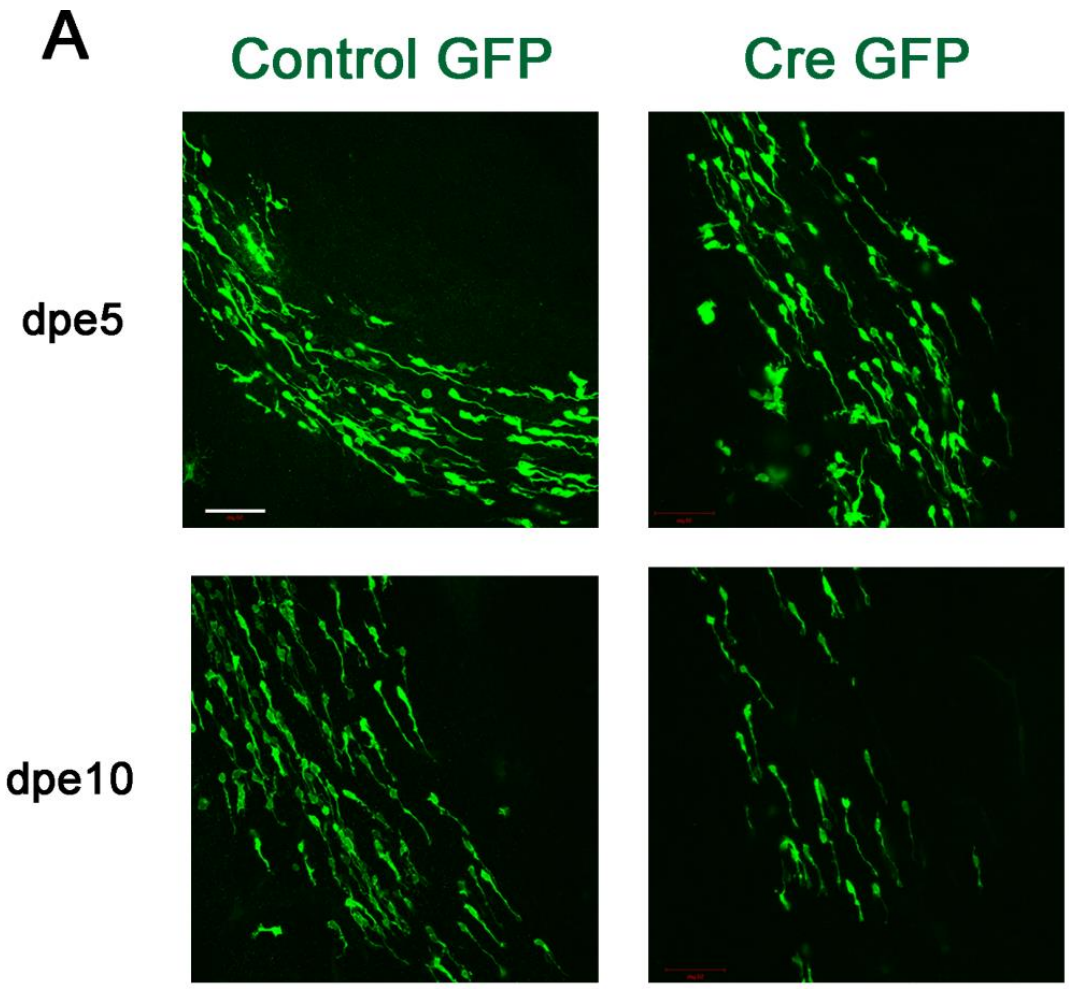


Figure 4-9 FGF-2 and FGFR1 transcript levels in the SVZ and OB of the postnatal mice

Brains from P7 mice pups were sliced and dissected for RNA extraction followed by RT-qPCR analysis. A sagittal section with coronal view of relative dissected area of a postnatal mouse forebrain (A, adapted from (Chiaramello et al, 2007). Red areas on coronal sections indicate two regions that were micro dissected for mRNA extraction. RT-qPCR results showing the expression levels of FGF-2 and FGFR1 compared to internal control GAPDH using Taqman gene expression assay (B). Transcripts encoding FGF-2 and FGFR1 were detectable both in the SVZ and the OB. However, the expression levels of FGF-2 were lower in the OB compared with the SVZ. Graph shows mean ± SEM (n=4, numbers of RT-qPCR experiments. 1-2 brains were used per dissection); **P<0.01, Two-way ANOVA followed by Bonferroni post-tests.

4.2.9 Neuroblast migration in the RMS is not obviously altered in mice lacking both the FGFR1 and FGFR2

The results above using acute perturbation of FGF-2/FGFR signalling support the hypothesis for these moleculses regulating neuroblast migration in the RMS. We next tested if conditional deletion of both FGFR1 and 2 would result in disruption of neuroblast migration in the stream. Conditional Fgfr1/2 knock-out mice employing the Cre-LoxP system are extremely useful for studying FGFR1/2 function as both genes can be deleted from the genome by introducing Cre recombinase (Chakkalakal et al, 2012; Xu et al, 2002; Yu et al, 2003; Yu et al, 2011). In the present study I have attempted to remove both receptors from the proliferating neuroblasts in the SVZ by electroporating a Cre-containing plasmid into the lateral ventricle of Fgfr1/2 $^{flox/flox}$ mice. Briefly, Fgfr1 and Fgfr2 conditional knock out mice lines were crossed to generate Fgfr1/2 $^{flox/flox}$ mice line and tail DNA preparations were subsequently genotyped by PCR (Xu et al, 2002; Yu et al, 2003; Yu et al, 2011). They were bred and genotyped by Dr Kieran M. Jones (Dr M. Albert Basson group, Department of Craniofacial Development and Stem Cell Biology, King's College London). This mouse line was maintained on a mixed genetic background and only mouse pups which have both homozygous Fgfr1 $^{flox/flox}$ and Fgfr1/2 $^{flox/flox}$ alleles confirmed by genotyping were used. P2 Fgfr1/2 $^{flox/flox}$ mouse pups were electroporated with either a control GFP or pCAG-Cre-IRES-GFP (to conditionally knock out Fgfr1/2 genes) plasmids, brains were fixed, sliced and stained for GFP 5 or 10 days later. Representative images of migrating neuroblasts in animals electroporated with control GFP or pCAG-Cre-IRES-GFP in the descending arm of RMS are shown in Figure 4-10A. Detailed analysis of the process length of the neuroblasts from the RMS is shown in Figure 4-10B. The results showed no significant difference on the process length of migrating neuroblasts in pCAG-Cre-IRES-GFP electroporated groups, either 5 days or 10 days after electroporation (Figure 4-10B). Detailed analysis on process length in different regions of the RMS was shown in Figure 4-11. Again, no significant results were seen at either 5 days (Figure 4-11A) or 10 days (Figure 4-11B) after pCAG-Cre-IRES-GFP electroporation. Thus a treatment that should result in the conditional knock out of FGFR1/2 in the postnatal mice didn't obviously change the morphology of the migrating neuroblasts in the RMS and I will discuss these results in detail in the discussion below.


A
Control GFP
Cre GFP
dpe5
dpe10

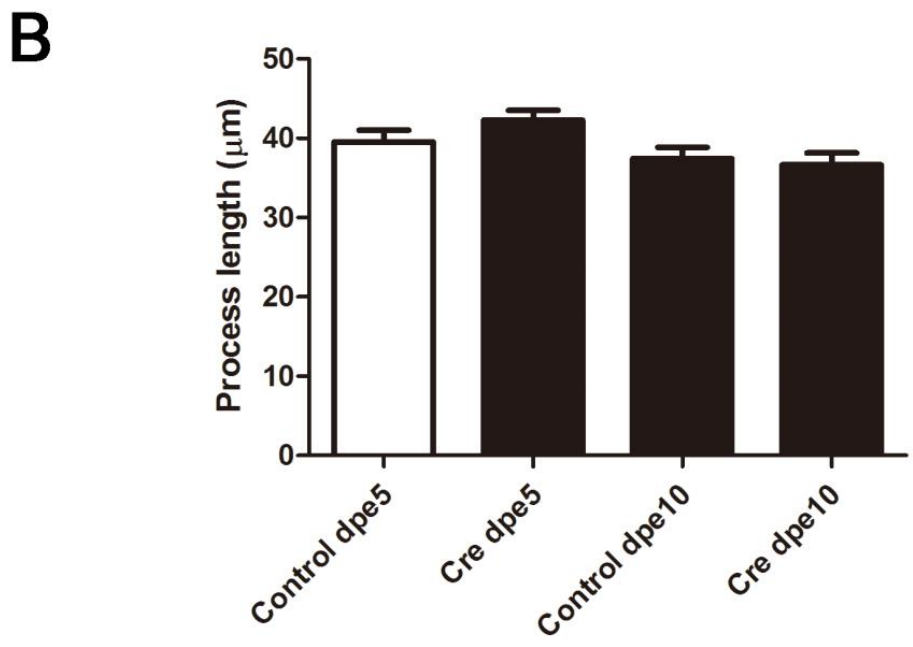

B
Process length (μm)
50
40
30
20
10
0
Control dpe5
Cre dpe5
Control dpe10
Cre dpe10

Figure 4-10 Conditional knock out of Fgfr1/2 in postnatal mice does not have a significant effect on neuroblast morphology

P2 Fgfr1/2 $^{flox/flox}$ mouse pups were electroporated with control GFP or pCAG-Cre-IRES-GFP plasmids, brains were fixed, sliced and stained for GFP 5 or 10 days later. Representative images of migrating neuroblasts in animals electroporated with control GFP or pCAG-Cre-IRES-GFP in the descending arm of RMS are shown in (A). Graph shows conditional knock out of FGFR1/2 does not have significant effect on the process length of migrating neuroblasts in the RMS (B). Graphs show mean ± s.e.m. (n= 2-3 animals for each group, 6 consecutive slices were analysed per brain); dpe, days post electroporation. Bar, 50 µm.

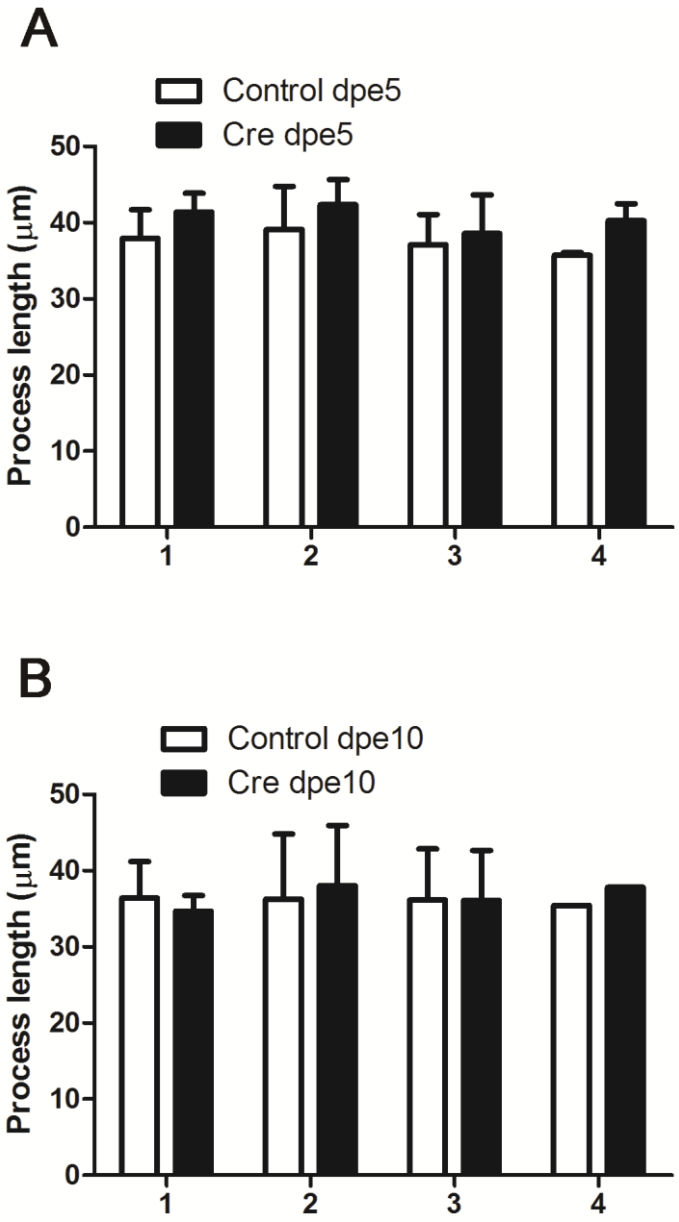


Figure 4-11 Conditional knock out of Fgfr1/2 in postnatal mice does not have a significant effect on neuroblast morphology in different regions of RMS

P2 Fgfr1/2 $^{flox/flox}$ mouse pups were electroporated with a control GFP or pCAG-Cre-IRES-GFP plasmid, brains were fixed, sliced and stained for GFP 5 or 10 days later. Graphs showing the quantification of process lengths of migrating neuroblasts in animals electroporated with control GFP and pCAG-Cre-IRES-GFP in 4 different regions along the RMS (labelled 1-4 as depicted in the cartoon in Figure 4-8) of dpe5 (A) and dpe10 (B). Results show conditional knock out of FGFR1/2 does not have significant effect on the process length of migrating neuroblasts along the RMS. Graphs show mean ± s.e.m. (n= 2-3 animals for each group, 6 consecutive slices were analysed per brain).

4.2.10 ROSA26-YFP mice for prolonged labelling of neuroblast in the RMS

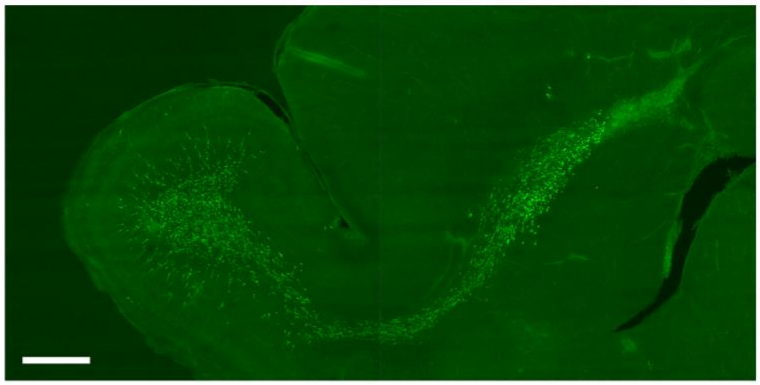
Neurogenesis in the SVZ is a life-long process in mammals and the neuroblast migration in the RMS persists to the adulthood, at least in rodents (Ming & Song, 2011). The results above were all obtained from young postnatal mice, and future studies will be required to test if the FGFR signalling is also required for neuroblast migration in the RMS in adult mice. In this study I wanted to do some preliminary experiments to determine the feasibility of establishing a model that would allow for detailed studies on migration within the RMS in young adult animals. The paradigm that we have used so far is limited to the study of young postnatal animals by the fact that the GFP expressing plasmids expressed in the P2/3 SVZ progenitors cells are realtively short-lived due to dilution within the rapidly proliferating progenitor cells. The technique of electroporation into adult mice is very low-efficient (Barnabe-Heider et al, 2008) and unlikely to result in the labelling of sufficient numbers of migratory neuroblasts. Therefore better methods are needed to label the migratory neuroblasts in adult mice. A commonly used approach in adult neurogenesis study is transgenic mice employing the Cre-LoxP site-specific recombination system (Lacar et al, 2010). A transgenic mouse line, ROSA26-YFP, has been generated that contains an YFP reporter within the genome that is normally silent due to a Stop sequence flanked by LoxP under the ubiquitous Rosa26 promoter (Lacar et al, 2010; Platel et al, 2010). Genomic expression of the YFP can be induced by excising the Stop sequence using Cre recombinase. Thus electroporation of Cre-expressing plasmids into the SVZ of young ROSA26-YFP pups can induce the stable expression of YFP in stem cells and their progeny. For example, electroporation of a Cre-containing plasmids on young ROSA26-YFP mice has been reported to be able to label the cells throughout the RMS and OB in adult mice (Lacar et al, 2010; Platel et al, 2010). In the present study we have tested this strategy for prolonged labelling of neurobast in the RMS. P2 ROSA26-YFP mouse pups were electroporated with a pCAG-Cre-IRES-EGFP plasmid and 1, 2, 4 or 8 weeks later, brains were dissected out and fixed, sliced and immunostained for the reporter construct (Figure 4-12). Sagittal sections of the brain slices showing cells in the RMS electroporated with Cre plasmids stay labelled up to 8 weeks (6 weeks old mice is defined as young adult), and the labelled neuroblasts keep migrating towards OB and populate into neurons in the OB (Figure 4-12). Visual inspection of the labelled neuroblasts in different ages of the ROSA26-YFP mice revealed no obvious difference in the morphology of the cells. Importantly, labelled cells could be seen at both ends of the RMS even 8 weeks after the electroporation illustrating the continuous production of “genetically” labelled migratory neuroblasts into adulthood (Figure 4-13). Neurogenesis declines

rapidly with age (Kuhn et al, 1996; Ming & Song, 2011), and as expected there was a clear decline in the number of cells entering the stream in the older pups (Figure 4-13).Thus electroporation of ROSA26-YFP mice with a Cre-containing plasmid in postnatal mice can probably lead to life-long genetic labelling of neural stem cells and their progeny allowing for the study of neuroblast migration in adult mice.

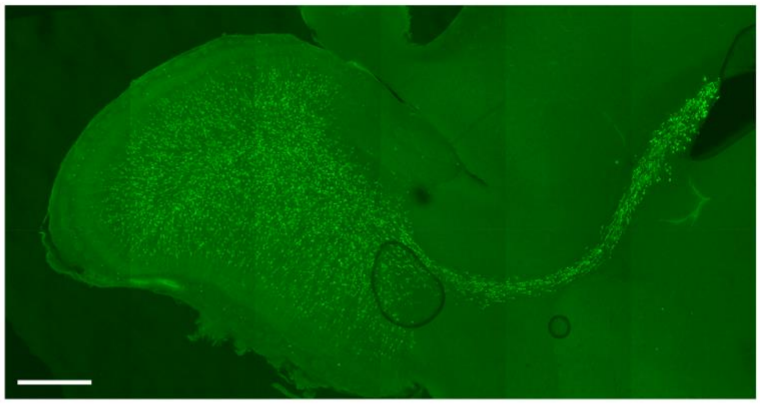
4.2.11 The effects of inhibiting FGFR signalling on neuroblast morphology in young adult mice

By using the above labelling paradigm in ROSA26-YFP mice, we conducted a preliminary study to test whether FGFR signalling regulates neuroblast migration in the RMS in young adult animals. P2 ROSA26-YFP mouse pups were electroporated with pCAG-Cre-IRES-EGFP and 6 weeks later treated with the FGFR inhibitor AZD4547 (12.5 mg/kg I.P., two doses with a 12 hour interval) essentially as previously described for the studies on young animals. After 24 h of AZD4547 treatment, animals were sacrificed and brains were fixed, sliced and stained for YFP. Images show representative neuroblasts in animals treated with vehicle (Figure 4-14A) or AZD4547 (Figure 4-14B) in the descending arm of RMS. Visual inspection of AZD4547 treated neuroblast revealed that treated cells tended to have shorter process length and a more branched leading process (Figure 4-14B). However, although detailed quantitative analysis confirmed this trend, the difference did not reach statistical significance (Figure 4-14C, data pooled from the whole RMS). Nonetheless, these preliminary studies show that it is feasible to conduct migratory studies in the RMS in young adult animals, however, the studies would clearly have to be powered with more animals in order to rigorously test the function of the FGF (and other) receptors.

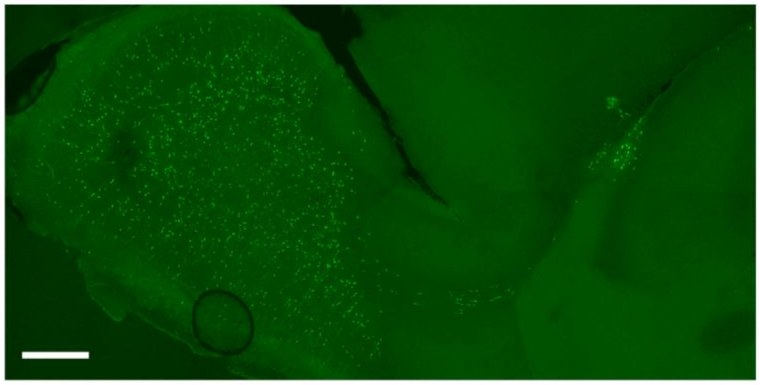
1 week



2 weeks



4 weeks



8 weeks

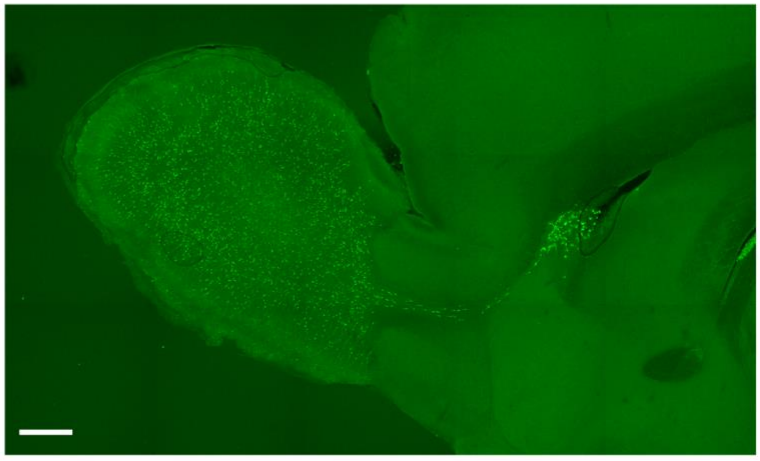


Figure 4-12 ROSA26-YFP mice for prolonged labelling of neuroblast in the RMS

P2 ROSA26-YFP mouse pups were electroporated with pCAG-Cre-IRES-EGFP and 1, 2, 4 or 8 weeks later, mice were sacrificed, brains were fixed, and slices containing the SVZ and RMS were immunostained for YFP. YFP positive cells are detectable at up to 8 weeks. Sagittal sections showing the labelled neuroblasts keep migrating towards OB and populate into neurons in the OB up to 8 weeks. Bar 500 μm.

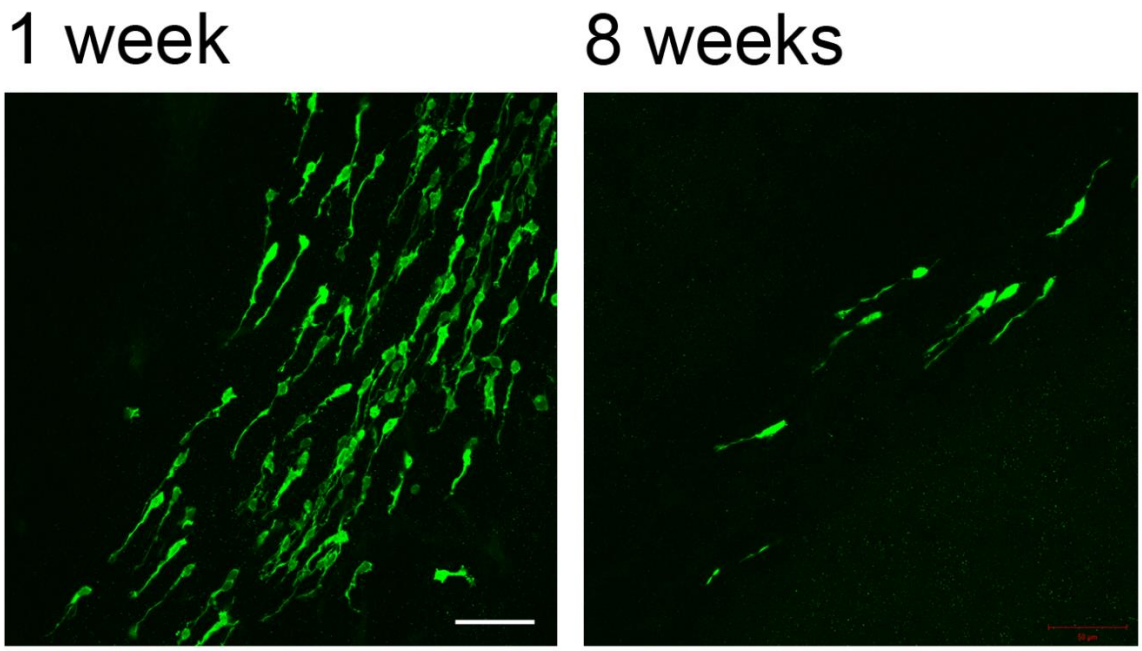


Figure 4-13 The morphology of neuroblasts in the RMS is not obviously different in 1 week old and 8 week old ROSA26-YFP mice

P2 ROSA26-YFP mouse pups were electroporated with pCAG-Cre-IRES-EGFP and 1 week or 8 weeks later, brains were fixed, sliced and stained for YFP. Representative images showing neuroblasts in the descending arm of RMS after 1 week (left) or 8 weeks (right) of electroporation. The number of labelled neuroblasts was reduced at 8 weeks due to the reduced rate of neurogenesis in the adult mice. However, cells in both groups exhibit typical migrating neuroblast morphology with a single leading process pointing towards the OB. Visual inspection of the neurobalst morphology reveals no significant difference between these two groups. Bar, 50 μm.

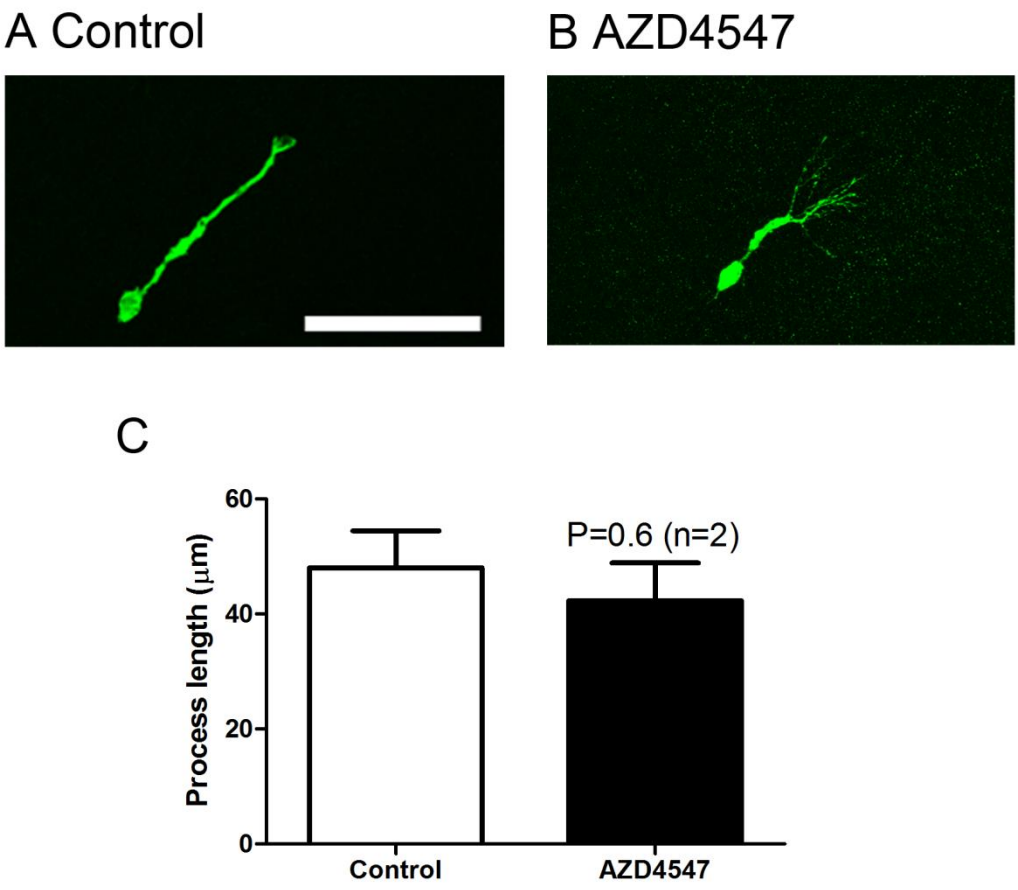


Figure 4-14 Preliminary data showing inhibiting FGFR signalling affects neuroblast morphology in young adult mice

P2 ROSA26-YFP mouse pups were electroporated with pCAG-Cre-IRES-EGFP and 6 weeks later treated with the FGFR inhibitor AZD4547 (12.5 mg/kg I.P., two doses with a 12 hour interval). After 24 h, brains were fixed, sliced and stained for YFP. Images show representative migrating neuroblasts in animals treated with vehicle (A) and AZD4547 (B) in the descending arm of RMS. AZD4547 treated neuroblasts tend to have shorter process length and branched leading process. Inhibiting FGFR signalling has a trend to decrease the process length of migrating neuroblasts in the RMS (C, data pooled from the whole RMS). Graphs show mean ± s.e.m. (n= 2 animals for control and drug treated, 6 consecutive slices were analysed per brain, ~50 cells analysed per brain); Bar, 50 μm for (A-B).

Chapter 5 TrkB signalling is required for directed cell migration within the RMS

5.1 Introduction

In the last chapters, we used time-lapse imaging to characterise the role of eCB and FGFR signalling systems in the regulation of neuroblast migration and found both similarities and differences between these two systems regulating migration in different regions of the RMS. The eCB signalling regulates neuroblast motility and guidance all along the RMS while the FGFR system is important at the beginning but not end of the RMS. However, by comparing the effects of these two systems at the beginning of the RMS, we didn't see any particular 'rank' of these two systems in regulating neuroblast migration. To further compare the relative importance of the eCB signalling to other putative signalling systems that govern neuroblast migration in the RMS, we have also treated the brain slices with tools to perturb TrkB signalling. TrkB or Tyrosine receptor kinase B is the main receptor for brain-derived neurotrophic factor (BDNF) (Acheson et al, 1995; Huang & Reichardt, 2001; Yamada & Nabeshima, 2003). Effects of BDNF/TrkB signalling in SVZ neurogenesis and RMS neuroblast migration have been reported previously (Bath et al, 2008; Chiaramello et al, 2007; Snapyan et al, 2009). However, controversial results have been reported on the role of BDNF/TrkB signalling in regulating neuroblast migration (Bagley & Belluscio, 2010; Snapyan et al, 2009), therefore it was interesting to re-investigate the role of this signalling system on neuroblast migration along the entire intact stream.

As mentioned in chapter 3, there is lots of evidence for direct cross-talk between the TrkB, its ligand BDNF and eCB signalling (Aso et al, 2008; Berghuis et al, 2005; Blazquez et al, 2015; Lemtiri-Chlieh & Levine, 2010; Maison et al, 2009). Previous studies also show that BDNF and its receptor - TrkB are expressed throughout the SVZ-OB system, also BDNF has a motogenic effect on neuroblasts in RMS explant cultures (Chiaramello et al, 2007), indicating an important role for BDNF and TrkB in the RMS. However, conflicting studies have appeared on the role of BDNF signalling in the RMS. For example, blocking BDNF function with an antibody has been reported to increase both the speed of migration and the number of neuroblasts migrating towards the OB (Bagley & Belluscio, 2010). However, another study found that neuroblast migration in acute brain slices from adult mice is inhibited by incubation with a TrkB-Fc that in principle should act in a similar way to a BDNF blocking antibody (Snapyan et al, 2009). To try to clarify this issue we have

revisited this question by determining the effect of a TrkB-Fc on neuroblast motility and guidance along the stream.

The TrkB-Fc is a chimeric molecule consisting of a human Fc fused to the ligand binding domain of the neurotrophin receptor; it will bind the TrkB ligands BDNF and NT4 and prevent them from interacting with the cell expressed receptors (Binder et al, 1999; Snapyan et al, 2009). Our results suggest that TrkB signalling does indeed regulate neuroblast migration throughout the stream. By comparing with the slices treated with the eCB inhibitors, the treatment with TrkB-Fc was seen to have a remarkably similar effect on the neuroblast migration in the RMS since it disrupts both motility and guidance, and it operates along both ends of the RMS. Non-specific effects of the TrkB-Fc can be excluded based on the observation that a TrkA-Fc had no significant effect on neuroblast migration in the RMS.

5.2 Results

5.2.1 A TrkB-Fc perturbs neuroblast migration in the RMS

In order to test the importance of the TrkB receptor for neuroblast migration in the RMS, we added a TrkB-Fc to the cultured brain slices as this reagent will bind BDNF, and NT4, and prevent them from interacting with TrkB (Binder et al, 1999; Snapyan et al, 2009). P2 mice pups were electroporated with pCX-EGFP to label stem cells in the ventral wall resulting in the presence of GFP-labelled neuroblasts within the stream 5-7 days later. Slices were equilibrated for 2 h in control medium or medium containing TrkB-Fc (1 µg/ml) before being imaged for 3 h. In our initial analysis we focused our attention on the descending arm of the RMS before the “elbow” region (region 1 in Figure 5-3). Individual neuroblasts were tracked and migration analysed. Representative images of migrating neuroblasts, tracked for 3 hrs, for control and TrkB-Fc-treated slices are shown in Figure 5-1A,B. The red and blue arrows highlight the position of two neuroblasts in each condition over the 3 h period. Control neuroblasts migrated over longer distances towards the OB compared to neuroblasts in slices treated with the TrkB-Fc (Figure 5-1A,B). In addition, control cells often display a predominant unipolar morphology oriented towards the OB, while cells treated with TrkB-Fc displayed branched processes extending in all directions (Figure 5-1A,B). The migration tracks of individual neuroblasts from representative slices are shown in Figure 5-1C-D. Under control conditions neuroblasts tended to follow a similar path towards the OB (Figure 5-1C), while the cells in the slices treated with TrkB-Fc clearly migrate in a less directed manner (Figure 5-1D).

A detailed statistical analysis of cell migration dynamics in these experiments is summarised in Figure 5-2A-C. In control slices, neuroblasts were immobile (nucleus moved less than 2 μm) ~22% of their time, but this increased to ~31% ($p<0.01$) in the presence of TrkB-Fc (Figure 5-2A). In control medium, ~70% of neuroblasts show net migration towards the OB (Figure 5-2B), and this was significantly reduced by treatment with the TrkB-Fc to around 40% (Figure 5-2B). Moreover, total cell displacement was also substantially reduced (103.0 ± 6.4 μm for control and 72.9 ± 2.9 for treated) (Figure 5-2C). There was a relatively small effect on the average speed of TrkB-Fc-treated cells (control 61.2 ± 1.8 μm/h, and TrkB-Fc 51.6 ± 2.7 μm/h, $P<0.05$). In summary, based on the measurement above, the results clearly support the hypothesis that TrkB signalling regulates neuroblast migration in the intact RMS.

5.2.2 A TrkB-Fc perturbs directed cell migration at both ends of the RMS

Next we wanted to address the important question as to whether TrkB is important at both ends of the stream. To quantitatively examine the effect of inhibiting TrkB signalling on the directionality of neuroblast migration towards the beginning and end of the RMS, again we analysed populations of neuroblasts sampled towards the beginning or end of the RMS as indexed by region 1 and region 2 in Figure 5-3A. Again we monitored the directionality of neuroblast migration by calculating the “meandering” index, i.e. the ratio between net displacement and total distance covered over the 3 h time period. Our results showed that in the RMS, close to the SVZ (Figure 5-3A), treatment with TrkB-Fc resulted in a significant reduction in the number of neuroblasts showing directed migration with significant increase of exploratory neuroblasts. And importantly this was apparent at both ends of the stream (Figure 5-3). Thus, we can conclude that TrkB signalling is required for neuroblast motility and guidance within the RMS and that this pathway operates all along the RMS.

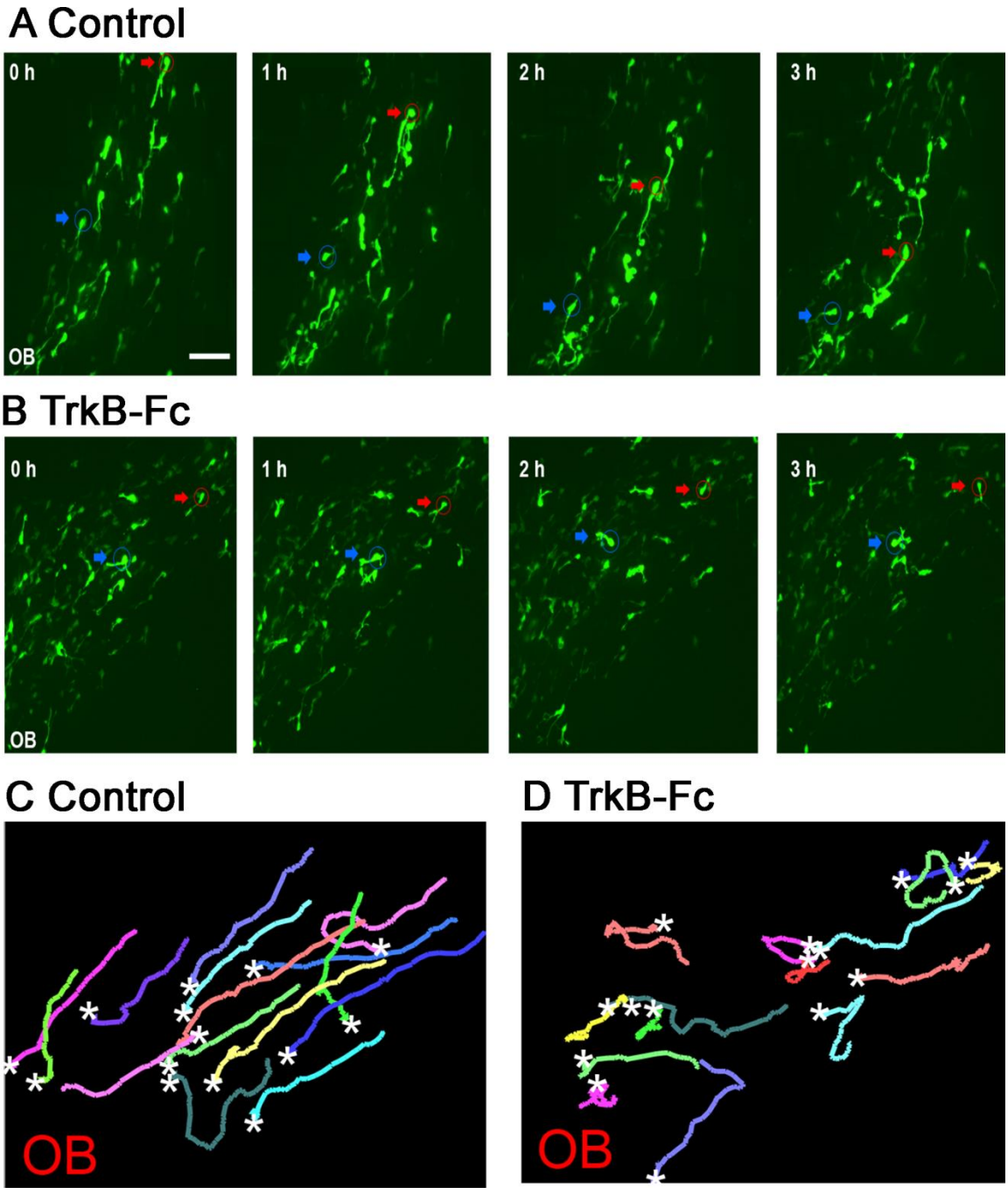

A Control
0 h
1 h
2 h
3 h
OB
B TrkB-Fc
0 h
1 h
2 h
3 h
OB
C Control
D TrkB-Fc
OB
OB

Figure 5-1 A TrkB-Fc perturbs neuroblast migration in the RMS

Sagittal mouse brain slices with GFP-labeled neuroblasts were prepared 5-7 days after *in vivo* postnatal electroporation of P2 mice with pCX-EGFP, cultured with vehicle or a TrkB-Fc for 2 hours and subsequently imaged for 3 hours in the same medium. Time-lapse movies made from the descending arm of the RMS in slices treated with a TrkB-Fc at 1 µg/ml were analyzed using Volocity. Representative pictures of slices treated with vehicle (A) or the TrkB-Fc (B) are shown. Arrows follow two neuroblasts in each frame. Representative migratory tracks of 15 cells over a 3 hour period from a control (C) and a TrkB-Fc-treated brain slice (D). White stars mark the end point of each migration track. OB shows the location of the olfactory bulb in each brain slice. Cells migrate towards OB with time in control slice while TrkB-Fc treated cells show less directed movements. Bars, 70 µm for (A–B).

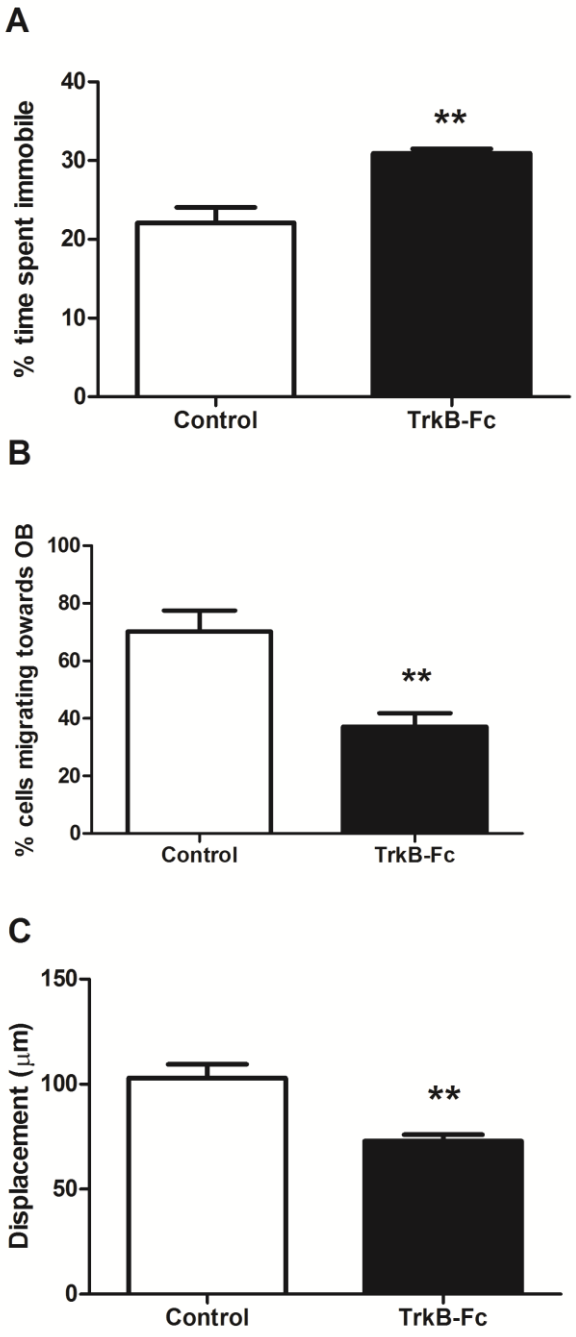


Figure 5-2 The TrkB-Fc perturbs neuroblast migration in the RMS

Quantification of cell migration from control slices or slices treated with a TrkB-Fc (1 µg/ml). Time-lapse movies all made from the descending arm of the RMS. Treatment with the TrkB-Fc increased the percentage of time neuroblasts spent immobile (A). Incubation with the TrkB-Fc also significantly decreased the percentage of neuroblasts migrating towards the OB (B) and the overall cell displacement (C). Graphs show mean ± s.e.m. (n=7 brain slices for each condition, ~ 15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

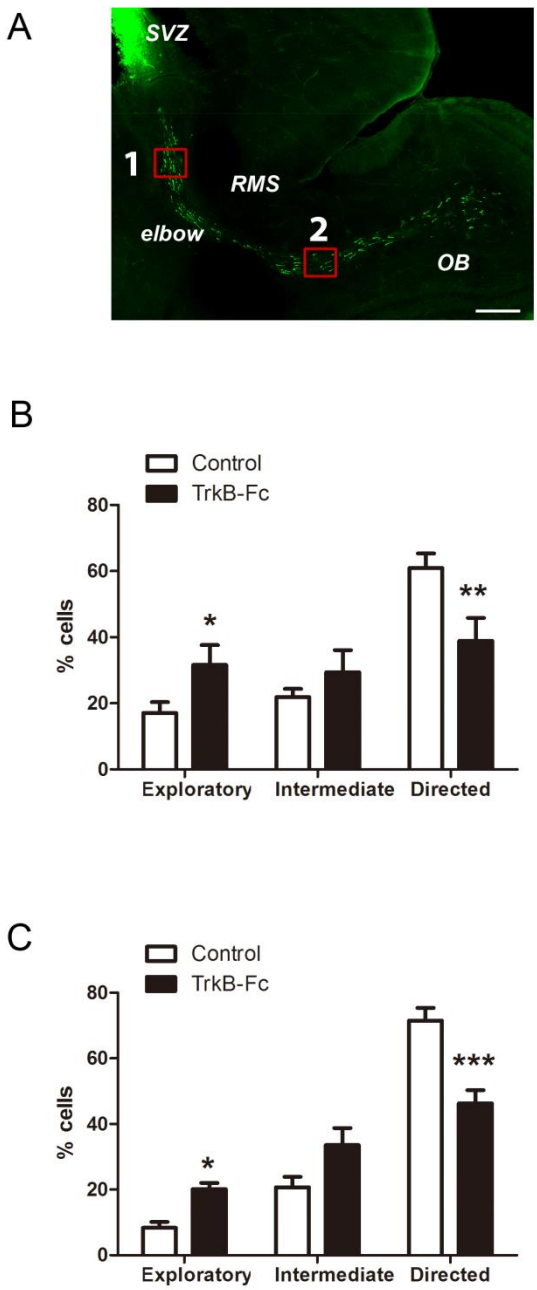


Figure 5-3 TrkB-Fc perturbs neuroblast directionality along the RMS

Five days after *in vivo* electroporation of pCX-EGFP in P2 mice, brain slices were cultured with vehicle or TrkB-Fc (1 µg/ml) for 2 h followed by time-lapse imaging for 3 h in the same medium. Neuroblast migration was filmed and analysed at the beginning and end of RMS (regions 1 and 2 in A). Incubation with the TrkB-Fc signifiantly increased the percentage of exploratory neuroblasts at the expense of directed neuroblasts towards the beginning (B) and at the end of the RMS (C) compared to control slices. Graphs show mean ± s.e.m. (n=6-8 brain slices for each condition, ~15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.2.3 A TrkA-Fc does not significantly affect neuroblast migration in the RMS

The above results provide clear evidence of an important role for the TrkB receptor in the regulation of neuroblast migration within the RMS. Previous studies have indicated that the BDNF and its TrkB receptor are expressed throughout the SVZ-OB system while the effect of the closely related TrkA receptor, which is responsive to NGF, on RMS neuroblast migration has not been reported (Berghuis et al, 2005; Chiaramello et al, 2007). To investigate a possible role for NGF/TrkA in neuroblast migration, we added a TrkA-Fc to the cultured brain slices. P2 mice pups were electroporated with pCX-EGFP and slice cultures containing GFP-labelled neuroblasts within the stream were established 5-7 days later. Slices were equilibrated for 2 h in control medium or medium containing TrkA-Fc (1 µg/ml) before being imaged for 3 h. Again, our initial analysis was focused on the descending arm of the RMS before the “elbow” region (region 1 in Figure 5-3A). Individual neuroblasts were then tracked and migration analysed. The migration tracks of individual neuroblasts from control and TrkA-Fc-treated slices are shown in Figure 5-4A,B. Visual inspection of the neuroblasts migration tracks in slices treated with the TrkA-Fc does not significantly differ from the control slices (Figure 5-4 A,B). Neuroblasts tended to follow a similar path towards the OB both in control and TrkA-Fc treated slices (Figure 5-4A,B). A detailed statistical analysis of cell migration dynamics in these experiments is summarised in Figure 5-4C-E. Neuroblasts were immobile (nucleus moved less than 2 µm) ~22% and 17% of their time in control and treated slices respectively ($P>0.5$) (Figure 5-4C). In control medium, ~70% of neuroblasts show net migration towards the OB (Figure 5-4D), and this was not significantly reduced by TrkA-Fc treatment (61%, $P>0.5$) (Figure 5-4D). Total cell displacement was also not significantly affected (103 ± 6.4 µm for control and 106 ± 10.0 µm for TrkA-Fc treated, $P>0.5$) (Figure 5-4E). Addtionally there was no significant effect on the speed of migration (61.2 µm/h for control and 67.1 µm/h for TrkA-Fc treated, $P>0.5$). In summary, the above results suggest that NGF and its receptor TrkA do not play a role in regulating neuroblast migration in the intact RMS

5.2.4 A TrkA-Fc does not affect directed cell migration at both ends of the RMS

Although the above results argue against a role for the TrkA receptor on neuroblast migration in the RMS, for completeness we also analysed populations of neuroblasts sampled towards the beginning or end of the RMS as indexed by region 1 and region 2 in Figure 5-3A. Again, we monitored the directionality of neuroblast migration by calculating the “meandering” index. Similar results were obtained in both regions of the stream when slices were treated with TrkA-Fc, and no

significant effects were observed by comparing to the control slices (Figure 5-5B,C). More specifically, close to the SVZ in the RMS (Figure 5-5A, region 1), treatment with TrkA-Fc resulted in ~24% of exploratory neuroblasts and ~45% of cells migrating in a directed manner, while in control slices, it was ~17% of exploratory and 60% directed, respectively and this does not reach statistical significance ($P>0.05$) (Figure 5-5B). Also, no significant effect was observed when neuroblasts were analysed in the RMS close to the OB (Figure 5-5C), treatment with TrkA-Fc resulted in ~11% of exploratory neuroblasts and ~63% of cells migrating in a directed manner, while in control slices, it was ~10% of exploratory and 70% directed, respectively ($P>0.05$) (Figure 5-5C). Thus, we can conclude that TrkA-Fc does not significantly affect neuroblast motility and guidance along the RMS. These results also serve as a good internal control for the TrkB results as non-specific effects of the Fc portion of the chimera can be excluded.

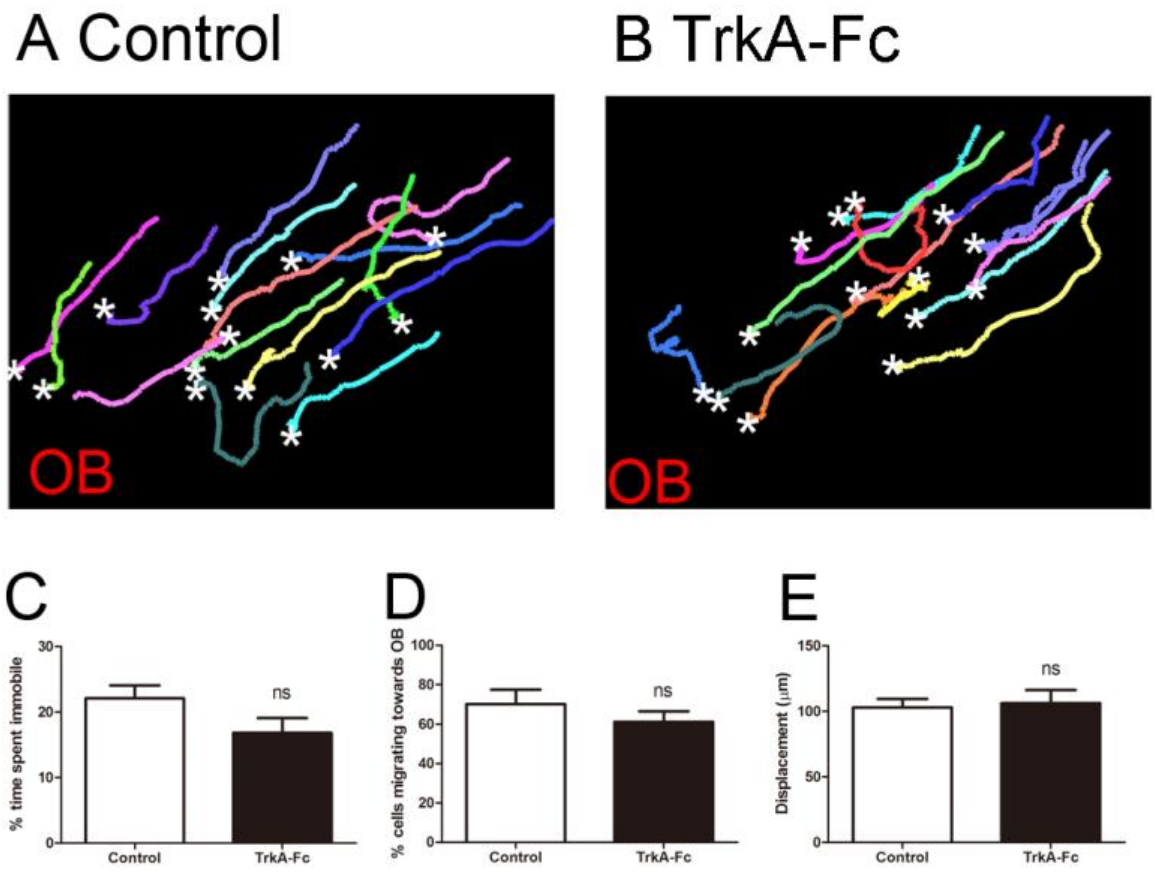


Figure 5-4 A TrkA-Fc does not significantly effect neuroblast migration in the RMS

Time-lapse movies made from the descending arm of the RMS in slices treated with a TrkA-Fc and were analyzed using Volocity. Representative migratory tracks of 15 cells over 3 hours from acontrol (A) or TrkA-Fc-treated (1 µg/ml) brain slices (B). White stars mark the tracking end point of each cell. The OB label shows the location of the olfactory bulb in each brain slice. Cells migrate towards OB with time in control and TrkA-Fc-treated slices. (C-E) shows quantification of cell migration from control slicesor slices treated with the TrkA-Fc. Treatment with TrkA-Fc has no significant effect on time spend immobile (C). Incubation with TrkA-Fc does not significantly decrease the percentage of neuroblasts migrating towards the OB (D) and the overall cell displacement (E). Graphs show mean ± s.e.m. (n=7 brain slices for each condition, ~ 15-30 cells analyzed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

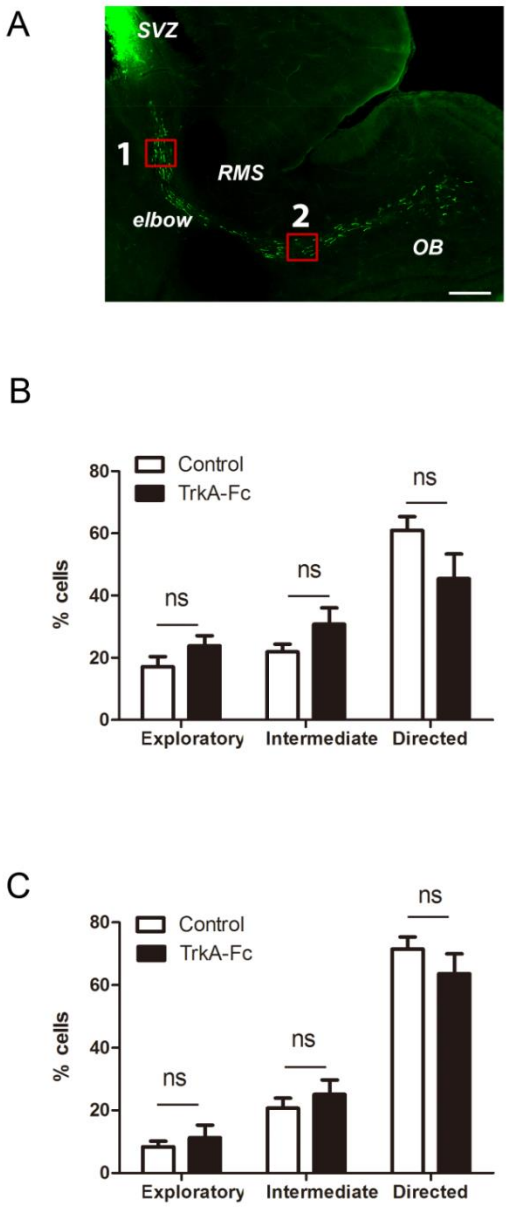


Figure 5-5 TrkA-Fc does not have significant effect on neuroblast directionality at both ends of the RMS

Five days after *in vivo* electroporation of pCX-EGFP in P2 mice, brain slices were cultured with vehicle or TrkA-Fc (1 µg/ml) for 2 h followed by time-lapse imaging for 3 h in the same medium. Neuroblast migration was analysed at the beginning and end of RMS (regions 1 and 2 in A). Incubation with the TrkA-Fc does not significantly affect the percentage of exploratory and directed neuroblasts towards the beginning (B) and end of the RMS (C) compared to control treatment. Graphs show mean ± s.e.m. (n=6-8 brain slices for each condition, ~15-30 cells analysed per slice); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Chapter 6 Intracellular trafficking of DAGLs

6.1 Introduction

In the last chapters we focused our attention on the role of the eCB and other signalling systems on neuroblast migration and established an important role for DAGL-dependent eCB signalling in the regulation of migration in the RMS. Considering that the DAGLs are also involved in axonal growth, guidance and synaptic signalling, I next turned my attention to the mechanisms that might govern the function of these key enzymes. As we mentioned in Chapter 3, the DAGLs are the key enzymes that produce 2-AG, the main endogenous ligand for the eCB receptors in the brain (Bisogno et al, 2003; Gao et al, 2010). Our lab first cloned these two enzymes and showed that they were expressed in the right place at the right time to regulate axonal growth and guidance as well as retrograde synaptic signalling (Bisogno et al, 2003; Gao et al, 2010). More specifically, DAGLs were shown to be expressed in axons during development, but the expression switched to and was restricted to dendrites in the adult brain (Bisogno et al, 2003). This switch in the expression patterns of DAGLs from axons to dendrites may correlate with a shift in their function from axonal growth and fasciculation to synaptic signalling at synapses (Bisogno et al, 2003; Oudin et al, 2011b; Yoshida et al, 2006).

Knock-out mice studies revealed that depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE), two related forms of short-term synaptic plasticity were lost in hippocampus, cerebellum and striatum in DAGLα-/- but not in DAGLβ-/- mice (Gao et al, 2010; Tanimura et al, 2010). These results suggested that DAGLα is responsible for retrograde eCB signalling in the CNS. Indeed, the restricted expression of DAGLα to dendritic spines in the adult brain was closely related to the pre-synaptic expression of CB1 in axon terminals (Bisogno et al, 2003; Oudin et al, 2011b; Yoshida et al, 2006). DAGLα is therefore perfectly placed in the right position to synthesis 2-AG to act as a retrograde messenger. This highly regulated and specific pre and post-synaptic localization of eCB components in the adult brain clearly points to the important role of eCB signalling in synaptic function (Oudin et al, 2011b). Therefore, understanding how the localization of DAGLs and other eCB components are regulated will be very important in understanding the eCB regulated synaptic plasticity.

Despite lots of studies that have been done on the function and cellular localization of DAGLs (Bisogno et al, 2003; Cecyre et al, 2014; Gao et al, 2010; Reisenberg et al, 2012; Yoshida et al,

2006), little is known about their regulation of localization within a cell. A previous study reported that DAGLα co-immunoprecipitated with Homer 2 in Neuro-2a cells (Jung et al, 2007). In this context Homer is a member of a family of scaffold proteins that help assemble molecular complexes in the post-synaptic density (Soloviev et al, 2000). Importantly, the interaction was abolished by mutating a consensus Homer-binding motif PPxxF in the C-terminal tail of DAGLα (Jung et al, 2007). Therefore Homer may play a role in DAGLα localization to dendritic spines in neurons. Interestingly, DAGLα can also directly interact with calcium/calmodulin-dependent protein kinase II (CamKII) (Shonesy et al, 2013). Co-immunoprecipitation of CamKII and DAGLα (but not DAGLβ) from mouse striatal extracts was reported (Shonesy et al, 2013). Activated CamKII can interact with the C-terminal domain of DAGLα by phosphorylation and inhibits its activity in vitro. Moreover, enhanced DSE was observed in a transgenic CamKIIα mice line which has reduced CamKIIα activity, indicating that CamKIIα is a negative modulator of short-term calcium-dependent eCB signalling (Shonesy et al, 2013). Another recent study reported that DAGLα mRNA can interact with fragile X mental retardation protein (FMRP), a protein involved in Fragile X syndrome, the most common inherited form of mental retardation (O'Donnell & Warren, 2002). This study also showed that normal perisynaptic co-localization of DAGLα and mGluR5 was impaired in *fmr1*-/- mice, a model for Fragile X syndrome which lacks FMRP production, suggesting that FMRP is necessary for the correct localization of DAGLα in dendritic spines (Jung et al, 2012). Moreover, blocking 2-AG degradation helped to correct key behavioural changes in *fmr1*-/- mice, indicating the localization of DAGLα in dendrites could be a therapeutic target for Fragile X syndrome (Jung et al, 2012).

Our motivation in studying DAGLs intracellular trafficking first comes from the fact that the DAGLs are localized to distinct cellular compartments during development and in adult (Bisogno et al, 2003; Yoshida et al, 2006). DAGLs are localized to axons during development, but are exclusively expressed in dendritic spines in the adult brain (Bisogno et al, 2003; Yoshida et al, 2006). This highly specific expression pattern is likely to be tightly regulated; however, the underlying mechanism is not known. One possible explanation could be the regulating of the intracellular trafficking or re-localization of the enzymes. Another fact is that 2-AG has a short half-life and does not diffuse over long distances (Rouzer et al, 2002), as the main enzymes for 2-AG synthesis, DAGLs must be localized to the right place at the right time to form an eCB signalling pool for effective signalling. Misallocated DAGLα at dendritic spine has been implicated in Fragile

X syndrome (Jung et al, 2012). Dynamic cycling of the receptor/enzyme is one of the most effective methods to regulate signal strength of the cell by regulating the rate of receptor/enzyme internalization, examples of this are AMPA and GABA receptor endocytosis at the synapses (Kittler et al, 2005; Kittler et al, 2000). Another reason is that DAGLα is expressed in close proximity to the CB1 receptor for efficient eCB signalling (Bisogno et al, 2003; Nyilas et al, 2009; Oudin et al, 2011b; Uchigashima et al, 2007; Yoshida et al, 2006). Constitutive endocytosis of the CB1 receptor has been reported previously and the pathway that mediates the CB1 receptor internalization is clathrin and dynamin dependent (Bohn, 2007; Hsieh et al, 1999; McDonald et al, 2007b). In the CNS, the CB1 receptor is well-positioned towards axons terminals to modulate synaptic signalling. Inhibition of CB1 receptor endocytosis by using a dominant-negative dynamin-1 (K44A) mutant disrupted this polarized localization by leading the cell-surface expressed GFP-CB1 onto somatodendeitic compartment (McDonald et al, 2007a). Like AMPA, GABA or CB1 receptors, DAGLα is also localised to the plasma membrane. Is DAGLα also subjected to endocytosis and dynamic cycling inside of the cell? To be able to follow DAGLα localisation in living cells I developed a new construct with an extracellular epitope tag which allows the surface labelling of DAGLα. By expressing this construct in neurons, we found evidence for intracellular and cell surface pools of DAGLα co-localised with Homer, a postsynaptic marker. A classical antibody feeding assay (Arancibia-Carcamo et al, 2006) revealed that DAGLα undergoes constitutive endocytosis in COS-7 cells and cultured hippocampal neurons. Detailed studies showed that DAGLα co-localizes with early endosome markers and undergoes recycling back to the cell surface in COS-7 cells. These novel findings have led us to postulate that dynamic cycling of DAGLα is likely to be a regulatory mechanism that will directly impact on eCB signalling at synapses and in other cellular contexts.

6.2 Results

6.2.1 Design of extracellular epitope-tagged human DAGLα constructs

The DAGLα protein consists of an intracellular N-terminal and four transmembrane domain (green), followed by a catalytic domain (pink) and a tail at the C-terminus (shown schematically in Figure 6-1). Since the N-terminal of DAGLα is intracellular, the way of simply inserting an epitope tag sequence into the N-terminal of the protein, the most commonly used way to enable surface labelling for GPCRs, is not applicable for live cell imaging. In principle, an epitope tag sequence can be inserted into one or other of the two extracellular loops of DAGLα. However, these two

extracellular loops are relatively small (20 and 15 amino acid residues respectively). In this context, the use of fluorescent protein tags which are relatively large proteins in themselves (for example GFP is composed of 238 amino acid residues) was not considered. The HA tag, a peptide sequence (YPYDVPDYA) derived from the human influenza hemagglutinin protein, was chosen by us because it's one of the smallest epitope tags available (nine amino acids in total) and high-affinity antibodies for western blotting, immunoprecipitation and especially immunofluorescence have been developed in many different species (Terpe, 2003). Also the presence of HA epitope tag has been reported not to interfere with the CB receptor internalization (Atwood et al, 2012). Because the two extracellular loops of DAGLα protein are relatively small, we added a linker sequence on each side of the HA sequence to provide flexibility for the recombinant regions to better ensure the expression of the epitope tag and the same strategy has been reported previously (Prakriya et al, 2006). Therefore, two constructs were designed, a HA peptide sequence (YPYDVPDYA) flanked by a glycine-serine-glycine-serine (GSGS) linker on either side was inserted into the first (Figure 6-1A) or second (Figure 6-1B) extracellular loop of human DAGLα between H and E (Figure 6-1A) or Y and T (Figure 6-1B) in pcDNA™6.2-DEST/V5 construct cloned by gateway cloning by Dr Praveen K Singh. The designed constructs will have an extracellular HA tag as well as an intracellular V5 tag located on the C-terminal of DAGLα.

6.2.2 Verification of the epitope-tagged human DAGLα constructs

The insertion of the HA sequence into DAGLα was confirmed by sequencing and western blot (data not shown). The constructs were then transfected into the COS-7 cell line to test if the constructs were expressed and to determine if anti-HA antibodies were able to recognise the epitope tag on the surface of living cells. Preliminary data pointed to the antibodies being able to interact better with the tag inserted into the first extracellular loop (Figure 6-1A and data not shown). Therefore this construct was used in the following studies and named as HA-DAGLα. The HA-DAGLα construct was transfected into COS-7 cells, 24 h later cells were fixed, permeabilized and stained for both HA and V5 tags. As expected, co-localization of these two epitope tags can be seen in the COS-7 cells (Figure 6-2A). We then wanted to examine if the HA tag can be successfully detected on the cell surface of live cells. The HA-DAGLα construct was transfected into various cell lines by FuGene transfection kit. 24 h later, a rat anti-HA antibody was added into the cell medium of live cells for 20 min at room temperature (RT); the cells were then gently

washed to remove unbound antibody and returned to the incubator for 30 min. Cells were then briefly fixed and an anti-rat 488 conjugated secondary antibody was used to label the anti-HA antibodies that associate with the surface pool of HA-DAGLα. Cells were then permeabilized with 0.2% TritonX-100 and a rabbit anti-V5 antibody was used and subsequently an anti-rabbit 594 was used to label the V5 tag associated with the total DAGLα pool. Images were taken by LSM710 confocal microscopy and all the images shown are sections through the middle of the cells (when the nucleus of the cell is the biggest). As shown in Figure 6-2B, this procedure revealed two pools of DAGLα; a cell surface pool labelled only with the anti-HA antibody and an intracellular pool detected by anti-V5 antibody. The surface expression of HA-DAGLα was also detected in HEK293 and Hela cells (data not shown). Thus we can conclude that the HA-DAGLα construct results in the expression of the enzyme in a manner that enables us to detect the extracellular epitope tag - it will therefore be a useful tool for a DAGLα trafficking study.

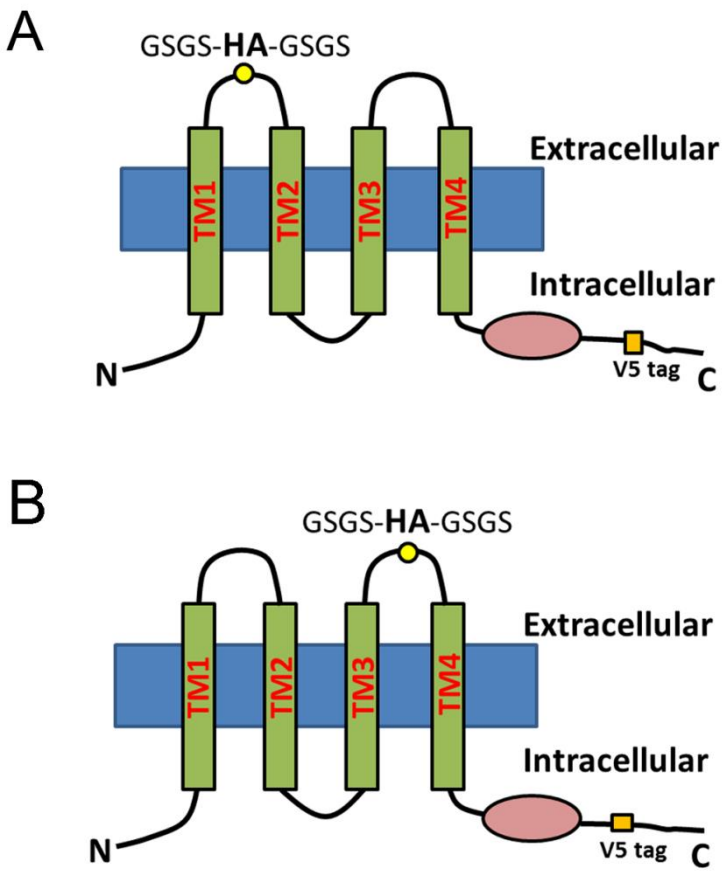


Figure 6-1 Design of extracellular epitope-tagged human DAGLα constructs

The DAGLα protein consists of an N-terminal and four transmembrane domains (green), followed by a catalytic domain (pink) and a tail at the C-terminus. To enable extracellular labelling of DAGLα, a HA peptide sequence (YPYDVPDYA) flanked by a glycine-serine-glycine-serine (GSGS) linker on either side was inserted into the first (A) or second (B) extracellular loop of Human DAGL alpha between H and E (A) or Y and T (B) in pcDNA™6.2-DEST/V5 construct cloned by gateway cloning by Dr Praveen K Singh.

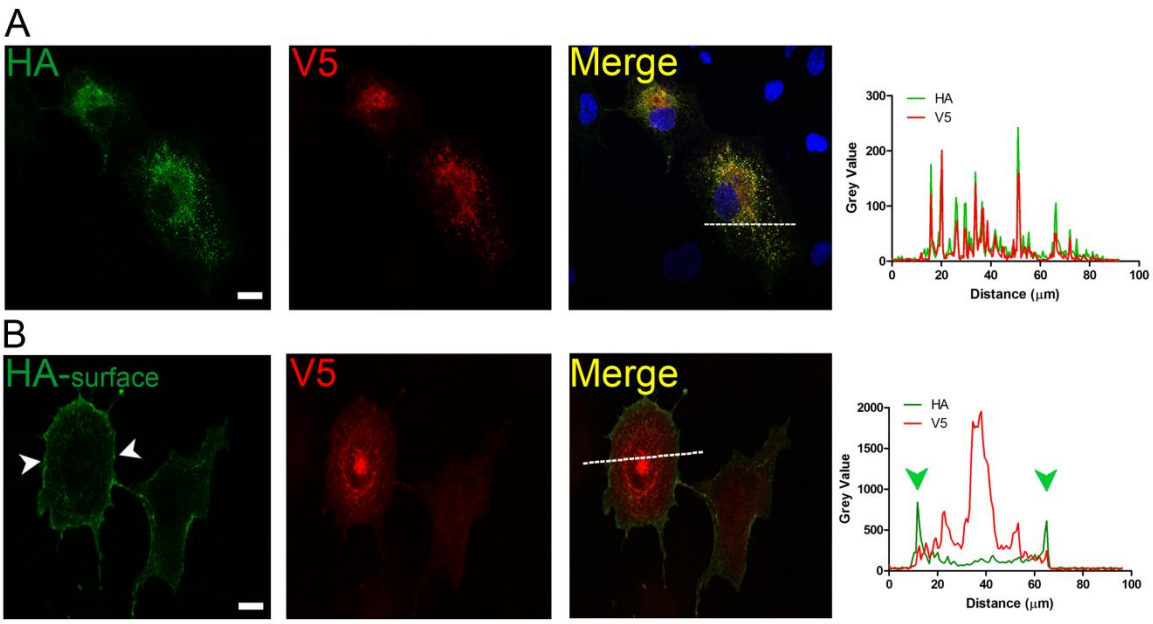


Figure 6-2 Expression of HA-DAGLα and surface labelling of HA tag in COS-7 cells

COS-7 cells transfected by HA-DAGLα were fixed, permeabilized and stained for HA and V5 tag. HA tag was successfully detected and shows co-localization with V5 tag in the COS-7 cells (A). Live cultures of COS-7 cells (B) transfected by HA-DAGLα were antibody fed with a rat anti-HA antibody for 20 min at RT. Cells were then washed and incubated at 37°C for 30 min and fixed, an anti-rat 488 conjugated antibody was used to detect the anti-HA antibodies associated with DAGLα on the cell surface. Cells were then permeabilized to stain for the total V5-DAGLα. Far right panel shows the intensity profiles along the dotted line drawn across the merged images. White arrows in (B) show the surface HA tag signalling which correspond to the green arrows in the right intensity profile graph. The HA tag was successfully detected in live cells and localised to the surface of COS-7 cells. All images shown are sections through the middle of the cells (same as in the following figures unless otherwise stated). Bar 20 μm.

6.2.3 DAGLα co-localizes with a dendritic marker, Homer, in rat hippocampal neurons

DAGLα has been reported to be expressed in dendritic spines in mature neurons including hippocampal neurons (Bisogno et al, 2003; Katona et al, 2006; Yoshida et al, 2006). A direct interaction between DAGLα and Homer 2, a dendritic marker has also been previously reported (Jung et al, 2007). We next wanted to express the HA-DAGLα in neurons to see the expression pattern of this construct. Hippocampal neurons were isolated from E18.5 rats and cultured *in vitro* for various time periods. The presence of synapses within our cultures was checked using commonly used synaptic markers between 10-14 days *in vitro* (DIV), the time points that synapse maturation has been reported to occur (Ichikawa et al, 1993; Kay et al, 2011). Staining for PSD-95, a post-synaptic marker (Jaffrey et al, 1998) and Bassoon, a pre-synaptic marker (Davydova et al, 2014) revealed punctate staining patterns, suggesting the presence of synapses during this time period (data not shown). Based on this, HA-DAGLα was transfected into 10 DIV cultures by NeuroMag Magnetofection system and 2 days later, the cultures were fixed and stained for the V5 epitope tag to label the total DAGLα pool as well as for total Homer (an anti-Homer antibody which recognises Homer 1/2/3 was used) (Soloviev et al, 2000) to label post-synaptic densities. The results are shown in Figure 6-3. Punctate patterns of staining were seen with both the anti-V5 and anti-Homer antibodies, and more importantly there was extensive co-localize between these DAGLα and Homer in the neuronal dendrites (Figure 6-3). Thus we can conclude that HA-DAGLα can be localised to the dendrites spines in cultured hippocampal neurons.

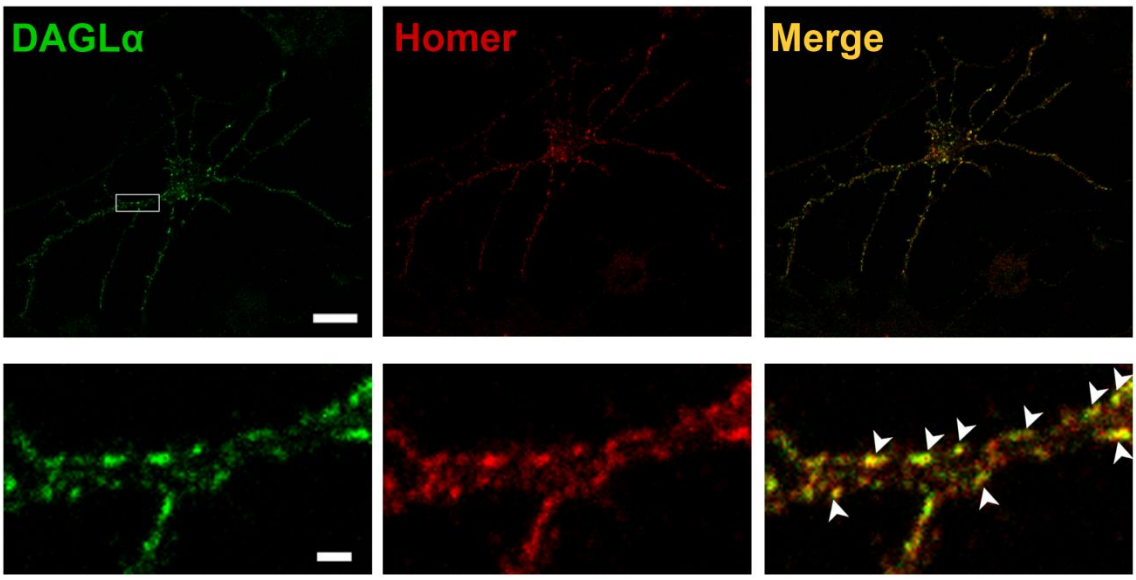


Figure 6-3 DAGLα co-localizes with Homer in rat hippocampal neurons

Rat hippocampal neurons were transfected with HA-DAGLα at 10 DIV using NeuroMag Magnetofection system and were fixed and permeabilized at 12 DIV. After blocking in 5% horse serum with 0.2%Triton X-100 for 20 min, an anti-V5 antibody and an anti-Homer antibody were used in the same medium to detect the V5 epitope tag as well as total Homer, a postsynaptic marker for neurons. Secondary antibodies with specific fluorophores were then used to differentiate total DAGLα pool (shown in green) and Homer (shown in red). The lower panel shows the zoomed in views of the white rectangular box areas. White arrows show co-localization of V5 and Homer. Extensive DAGLα co-localization with Homer in neuronal dendrites is clearly apparent. Scale bar: 10 µm and 1 µm for the zoom in.

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6.2.4 Live and permeabilized cell imaging reveals two pools of DAGLα in hippocampal neurons

Having determined that the HA-DAGLα construct can be successfully expressed on the dendrites of hippocampal neurons, we next wanted to determine if there are two pools of DAGLα in the dendrites - an intracellular pool and a cell surface pool. Rat hippocampal neurons were transfected by HA-DAGLα using NeuroMag Magnetofection system at 10 DIV and 2 days later, the rat anti-HA antibody was added to the live culture of the cells at room temperature to detect the HA epitope tag on the cell surface. 20 min later, neurons were washed and fixed. An anti-rat secondary antibody was used to detect HA epitope tag associated with surface DAGLα (shown in green in Figure 6-4). Cells were then permeabilized, an anti-V5 antibody was then used to stain for the total DAGLα pool (shown in red in Figure 6-4). Surface DAGLα labelled by HA tag co-localize well with total DAGLα labelled by V5 tag, and the surface DAGLα pool has a clear punctate pattern on dendrites (Figure 6-4). Intracellular pools of DAGLα that were only labelled with the anti-V5 antibody were also noticed (see white arrows in Figure 6-4), suggesting the existence of two distinct DAGLα pools in neuronal dendrites. Thus we can conclude there is a surface DAGLα pool on the dendrites of hippocampal neurons, and the HA-DAGLα construct can be used to differentiate surface from intracellular DAGLα in hippocampal neurons.

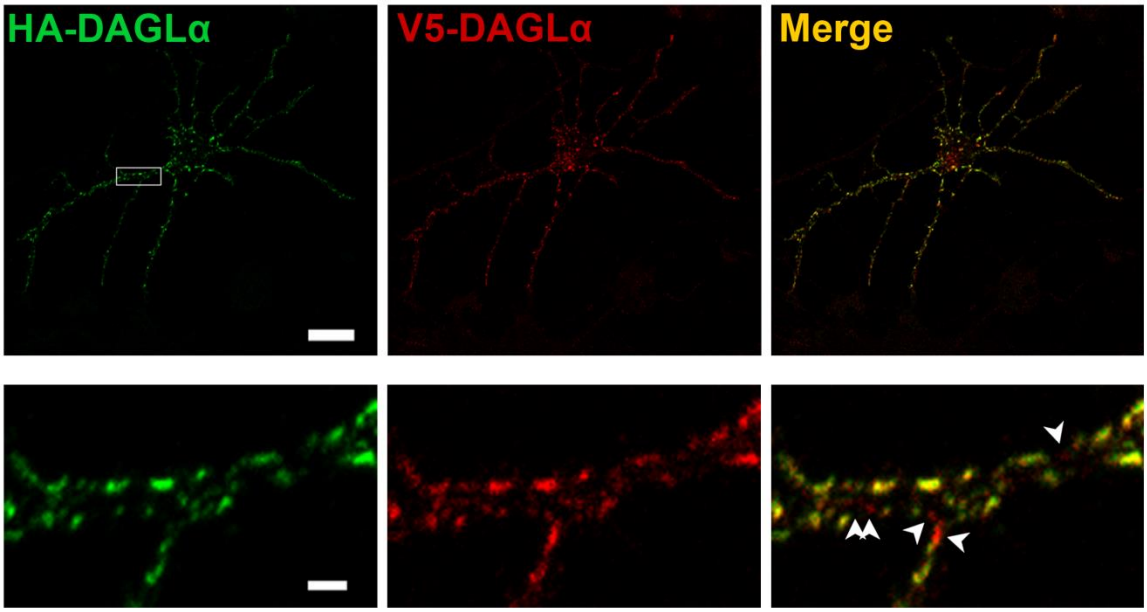
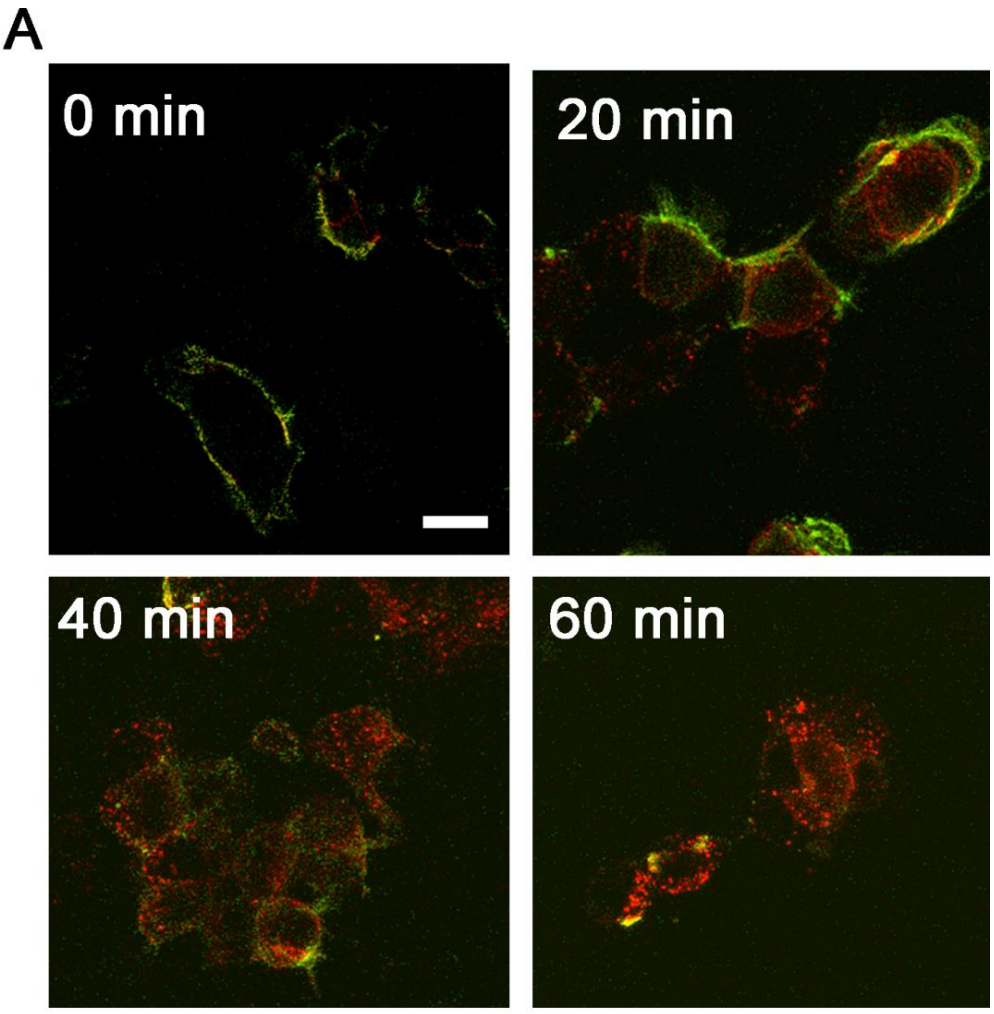


Figure 6-4 Live and permeabilised cell imaging reveals two pools of DAGLα in hippocampal neurons

The rat hippocampal neurons in Figure 6-3 were also live labelled for surface HA epitope tag for 20 min at room temperature. The rat anti-HA antibody was used for antibody feeding and unbound antibodies were washed out with buffered medium. Neurons were then briefly fixed and an anti-rat 488 secondary was used to detect the surface pool of DAGLα (shown in green). Cells were then permeablised and blocked in 5% horse serum with 0.2%Triton X-100 for 20 min. An anti-V5 antibody was then used to detect the total V5-DAGLα (shown in red). Surface DAGLα labelled by HA tag co-localizes well with total DAGLα labelled by V5 tag, and the surface DAGLα pool has specific punctate pattern on dendrites. The lower panel shows the zoomed in views of the white rectangular box. Note the presence of intracellular pools of DAGLα that only label with the V5-antibody – arrows in bottom right hand micrograph. Scale bar: 10 µm and 1 µm for the zoom in.

6.2.5 Time dependant internalization of the CB1 receptor in HEK293 cells

Having established that the HA-DAGLα construct is expressed appropriately in cells and at synapses, we next wanted to study the membrane trafficking of DAGLα. As a prelude we first determined if we could follow the internalization of the CB1 receptor since constitutive internalization of this GPCR has been previously reported, and this could then serve as a proof-of-principal experiment to validate our assay conditions (Hsieh et al, 1999; Osborne et al, 2009; Simon et al, 2013). To enable the surface labelling of the CB1 receptor, an N-terminal tagged CB1 construct, SEP-hCB1 was used (gift from Dr. Andrew J. Irving, University of Dundee) (McDonald et al, 2007b). SEP is a pH-sensitive variant of GFP that has similar fluorescence to wild-type GFP at physiological pH 7.4, but the fluorescent signal is reduced at acidic pH and completely quenched at pH 5.8 (the Golgi pH is 6.0 and endocytic vesicles pH is 5.5) (Demaurex, 2002; McDonald et al, 2007b; Miesenbock et al, 1998). Therefore, this N-terminal tagged SEP construct can be used to report surface expression of the CB1 receptor. We used an antibody feeding assay (Arancibia-Carcamo et al, 2006) to label the surface CB1 receptor with a rabbit anti-GFP antibody which recognizes the SEP tag, the cells were then allowed to internalize for various times before been fixed and an anti-rabbit 488 conjugated secondary antibody was used to detect the CB1 receptor that remained on the cell surface. Cells were then permeabilized and an anti-rabbit 594 conjugated secondary was used to detect the internalized CB1 receptor. When the CB1 receptor is internalized, red fluorescence (594 channel) should be detected under confocal microscopy and the rate of CB1 receptor internalization can then be measured by comparing the intensities of 594 (red) and 488 (green) channels. In this study, HEK293 cells were used and transfected with SEP-hCB1, 24 h later live cells were ‘fed’ with a rabbit anti-GFP antibody, cells were then returned back to cell incubator to allow receptor endocytosis to take place. Internalized CB1 receptor can be observed after 20 min of incubation (Figure 6-5A). Punctate, cytoplasmic vesicles accumulated inside of the cell after prolonged incubation (Figure 6-5A). Quantification of the intensity ratio between internalized and surface receptor revealed the time dependent, steady state constitutive internalization of the CB1 receptor (Figure 6-5B). These results were consistent with the previous studies on constitutive CB1 receptor internalization (McDonald et al, 2007a; McDonald et al, 2007b) and we can conclude that this antibody feeding and internalization assay can be used to study receptor/protein internalization.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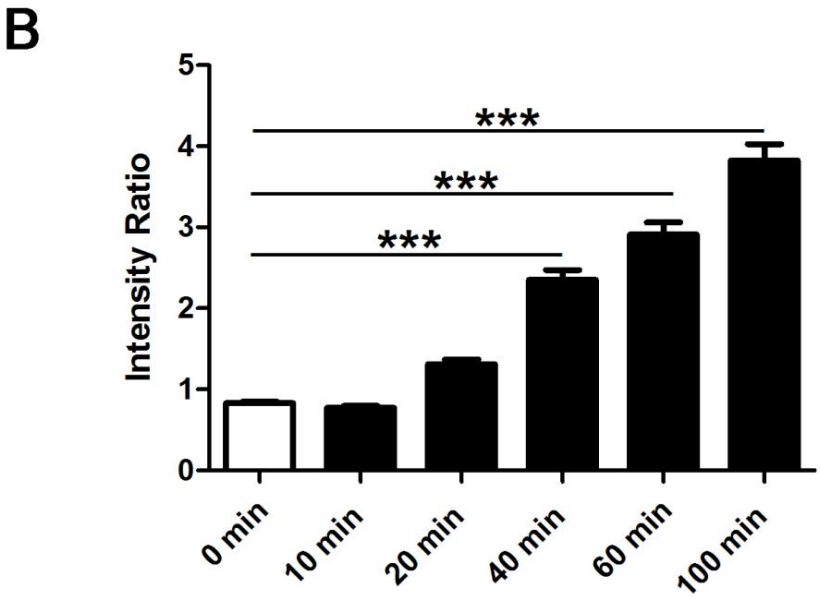
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10 min
20 min
40 min
60 min
100 min

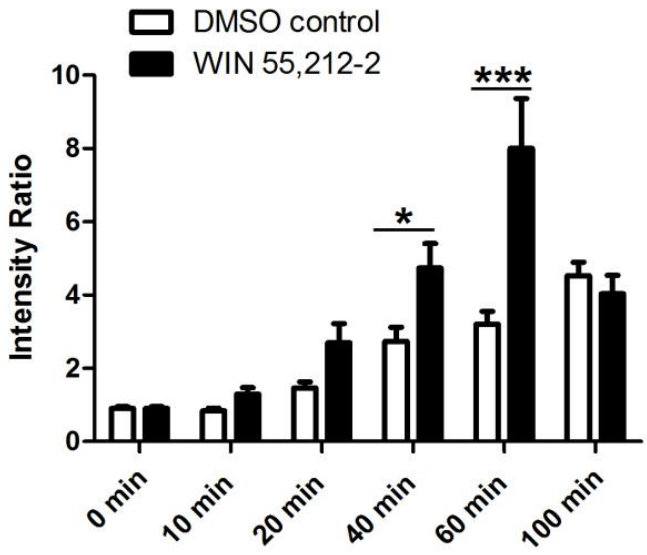
Figure 6-5 Time dependant internalization of the CB1 receptor in HEK293 cells

HEK293 cells expressing SEP-hCB1 were live labelled with a rabbit anti-GFP antibody for 20 min at 4°C, washed to remove unbound antibodies and then returned back to cell culture medium for 10 to 100 minutes at 37°C in the incubator. Cells were then fixed and incubated with an anti-rabbit 488 to label the remaining cell surface CB1 receptor. Cells were then permeabilized and incubated with an anti-rabbit 594 to label the internalized CB1 receptor. (A) Images show representative cells that were returned to the incubator for 20, 40 and 60 min at 37°C following the initial antibody labelling step. Constitutive internalization of the receptor (as revealed by the red-stain) can be seen from 20 min. All images were merged from individual images obtained in the green and red channels. Green channel, 488; red channel, 594. (B), Quantification of internalization by comparing the intensity ratio of 594 and 488 channel. Each bar represents the mean ± SEM (n=3 independent experiments, 10-15 fields were randomly taken from each coverslip, ~100 cells quantified for each group); *** $P < 0.001$. One-way ANOVA followed by Bonferroni post-tests. Scale bar: 20 µm.

6.2.6 Effects of CB1 agonists on the CB1 receptor internalisation

The internalization of the CB1 receptor has been reported to be induced by several CB receptor agonists, for example, WIN 55,212-2, a potent aminoalkylindole CB receptor agonist, can induce rapid CB1 receptor internalization at low concentration (30-100 nM) (Atwood et al, 2012; Hsieh et al, 1999). However, CB receptor agonists vary in their ability to cause internalization. For example, cannabinoids like anandamide (AEA) or tetrahydrocannabinol (THC) failed to increase the rate of CB1 receptor internalization at relatively high concentrations (>1500 nM) (Atwood et al, 2012; Hsieh et al, 1999). Therefore, in this study we wanted to reproduce these results to validate our internalization assay. WIN 55,212-2, a potent CB receptor agonist which also acts at CB2 receptor (Atwood et al, 2012) and ACEA, a selective CB1 receptor agonist which is an analogue of AEA were used (Hillard et al, 1999). HEK293 cells expressing SEP-hCB1 were live labelled for surface receptor by antibody feeding as previously described, and the cell cultures were then treated with either 1μM WIN 55,212-2 or a vehicle (0.1% DMSO) control. Cells were either fixed or returned back to the incubator for endocytosis for up to 100 min before fixation. Quantification of the internalization rate was determined as previously described and is shown in Figure 6-6A. Treatments with WIN 55,212-2 significantly increased the level of internalised CB1 receptor at 40 and 60 min. The treatment at 100 min was not significantly different from the control, and this is probably due to the degradation of the receptor after prolonged agonist activation (Hsieh et al, 1999). We next compared the effects of WIN 55,212-2 and ACEA (1μM each) by looking at internalization at 30 min after antibody feeding (again using 0.1% DMSO containing media as a vehicle control). The results shown in Figure 6-6B confirmed that WIN 55,212-2 can stimulate significant internalization of the CB1 receptor while ACEA has no significant effect. Thus our results are consistent with the previous studies that WIN 55,212-2 increases the internalization of CB1 receptor, whereas ACEA does not (Atwood et al, 2012; Hsieh et al, 1999).

A


DMSO control
WIN 55,212-2
Intensity Ratio
10
8
6
4
2
0
*
***
0 min
10 min
20 min
40 min
60 min
100 min

B

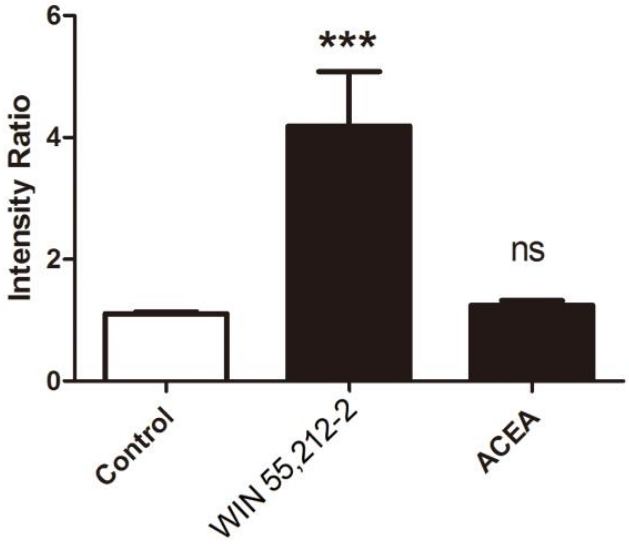

Intensity Ratio
6
4
2
0
***
ns
Control
WIN 55,212-2
ACEA

Figure 6-6 Effects of CB1 agonists on CB1 receptor internalisation

HEK293 cells expressing SEP-hCB1 were antibody fed with a rabbit anti-GFP antibody for 20 min at 4°C. Cells were washed and either fixed to give the 0 min time point control or returned back to cell culture medium with 1μM WIN 55,212-2 or 0.1% DMSO for up to 100 min in the 37°C incubator for uptake before fixation. Cells were then incubated with an anti-rabbit 488 to label the remaining cell surface CB1 receptor. Cells were then permeabilized and incubated with an anti-rabbit 594 to label the internalized CB1 receptor. Quantification of the internalized and surface intensity ratio is shown in (A), treatment with WIN 55,212-2 significantly increased the CB1 receptor internalization rate at 40 and 60 min compared to the DMSO control. (B) HEK293 cells were live labelled as above before being treated with 0.1% DMSO as control, WIN 55,212-2 or ACEA (all at 1μM) and incubated for 30 min in the incubator. WIN 55,212-2 induced significant internalization of the CB1 receptor when comparing to the control, while ACEA treatment had no significant effect on the CB1 receptor internalization. Each bar represents the mean ± SEM (n=2, 10-15 fields were randomly taken from each coverslip, ~100 cells quantified for each group); *<0.05; ** P < 0.01; *** P < 0.001. One-way or two-way ANOVA followed by Bonferroni post-tests.

6.2.7 DAGLα is constitutively internalized in COS-7 cells

DAGLα is highly localised to dendritic spines in mature neurons and it plays an important role in synaptic plasticity by regulating DSI and DSE (Bisogno et al, 2003; Gao et al, 2010; Tanimura et al, 2010). Dynamic cycling of membrane receptors, e.g. NMDA and GABA(A) receptors, has been reported to regulate synaptic strength (Arancibia-Carcamo et al, 2006; Kittler et al, 2000). Also the localization of DAGLα in dendritic spines is highly correlated with the pre-synoptically expressed CB1 receptor, which undergoes constitutive endocytosis in mature neurons (Cecyre et al, 2014; Oudin et al, 2011b; Uchigashima et al, 2007; Yoshida et al, 2006; Yoshida et al, 2011). Like NMDA and CB1 receptors, DAGLα is a transmembrane protein largely localised to the plasma membrane. We therefore tested if DAGLα is also subject to dynamic cycling at the cell surface. Results above have shown that there is a distinct surface pool that differs from the intracellular pool of DAGLα in COS-7 cells as well as hippocampal neurons. Therefore we postulated that DAGLα may also be internalized at the synapses in a dynamic manner. We first tested our hypothesis in COS-7 cells as their large cell size and ease of culture makes them a good model system for imaging and trafficking studies (Glebov et al, 2006; Mardakheh et al, 2009; Vandenbulcke et al, 2000). COS-7 cells were transfected with the HA-DAGLα construct using the FuGene transfection kit. 24 h later, the rat anti-HA antibody was added to the live cells for 20 min at 4°C. After antibody feeding, cells were washed and fixed to give a zero time point, or washed and returned to the incubator at 37°C for various times to allow endocytosis. Cells were then fixed and an anti-rat 488 secondary antibody was used to detect the anti-HA antibody present on the cell surface. Cells were then permeabilized and an anti-rat 594 antibody was used to label the anti-HA antibody associated with the internalized DAGLα pool. Confocal images were then taken under the same setting for all the time points. Results are shown in Figure 6-7. Visual inspection revealed that punctate red stained vesicles accumulate with time indicating that DAGLα is constitutively internalized in the COS-7 cells. Internalization was obvious from 20 min and the internalization level of DAGLα goes up with time during this incubation period at 37°C. Quantification of the internalization revealed a relatively steady state internalization of DAGLα with time (shown in Figure 6-8). Thus we can conclude that the DAGLα can be constitutively internalized in COS-7 cells.

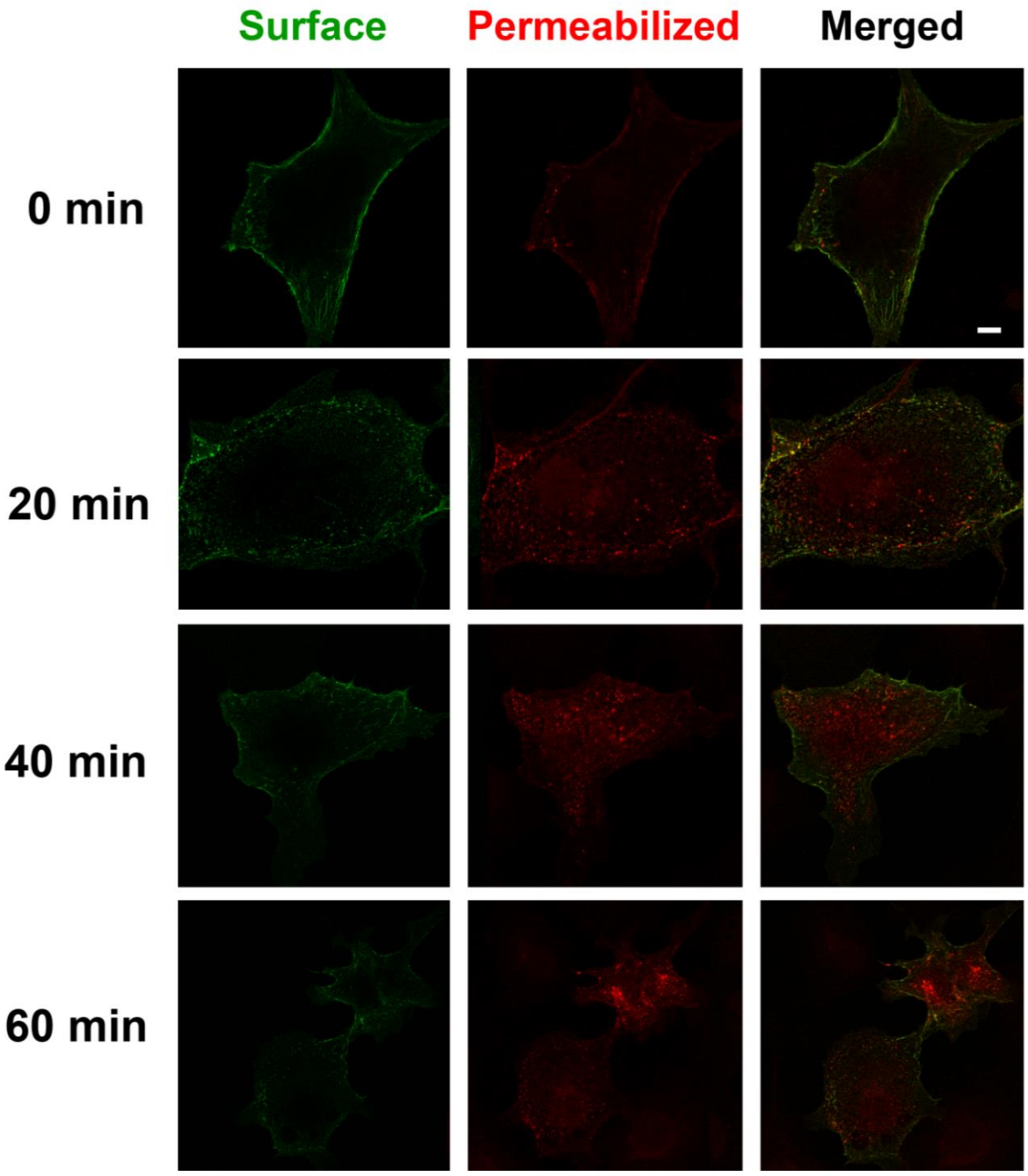

Surface
Permeabilized
Merged
0 min
20 min
40 min
60 min

Figure 6-7 DAGLα is constitutively internalized in COS-7 cells

COS-7 cells were transfected with the HA-DAGLα construct using the FuGene transfection kit. 24 h later, a rat anti-HA antibody was added to the live cells for 20 min at 4°C. Cells were then washed and fixed to give a zero time point, or washed and returned to 37°C incubator for 20 to 60 min to allow endocytosis. After incubation, cells were briefly fixed and an anti-rat 488 secondary antibody was used to detect the anti-HA antibody associated with DAGLα on the cell membrane. Cells were then permeabilized and an anti-rat 594 antibody was used to label the anti-HA antibody associated with the internalized pool of DAGLα. Confocal images were taken under the same setting for all the time points. Note the punctate red stained vesicles accumulates inside of the cell after incubation. Green channel, 488 to label surface HA-DAGLα; red channel, 594 to label HA-DAGLα after permeablization. Scale bar: 10 µm.

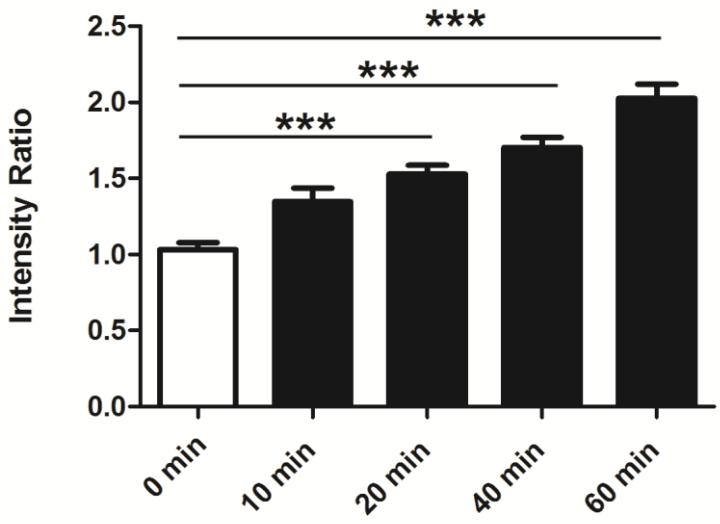


Figure 6-8 Quantification of DAGLα internalized in COS-7 cells

The 488 and 594 fluorophore intensities which were associated with surface and internalized DAGLα respectively were measured for the cells as shown in Figure 6-7. Intensity ratio (594 / 488) that indicates the rate of internalization of the cells was calculated and shown above. The results show DAGLα was significantly internalized after 20 min incubation at 37°C, the internalization level goes up with time to 60 min. Each bar represents the mean ± SEM (n=3, 10-15 fields were randomly taken from each coverslip, ~100 cells quantified for each group); *<0.05; ** P < 0.01; *** P < 0.001. One-way ANOVA followed by Bonferroni post-tests.

6.2.8 DAGLα is recycling in COS-7 cells

After showing that DAGLα undergoes constitutive internalization in COS-7 cells, we next wanted to know if the enzyme is simply internalised for degradation, or if it is recycled back to the cell surface. An “acid wash” or “antibody stripping” assay has been previously used to study the receptor internalization in neurons with the specific aim of seeing if a receptor is returned to the cell surface (Carroll et al, 1999; Hanley et al, 2002; Scott et al, 2004). By washing the cells at low pH before fixation, the assay can selectively strip any surface-bound primary antibody and, importantly, leave the internalized antibody-receptor complexes intact. If the complex is degraded in lysosomes the antibody will not re-appear on the cell surface. In contrast if the antibody reappears at the cell surface, it is taken as evidence that the antigen (in this case DAGLα) is in a pool that be regulated by dynamic vesicular cycling from and back to the cell surface.

The first important control in this assay is to ensure that the acid buffer can completely strip the surface bound antibody. COS-7 cells expressing the HA-DAGLα construct were live labelled with the rat anti-HA tag antibody for 30 min at RT, the cells were either washed and fixed directly or washed in a DMEM buffer (pH 3.0-3.5) before fixation (Figure 6-9a,b). Cells without the acid wash showed extensive labelling for the anti-HA antibody on the cell surface (Figure 6-9a). However, after acid wash, the surface staining was completely gone (Figure 6-9b), indicating the success of antibody stripping.

The next step was to test if the internalized antibody-enzyme complexes can still be detected after the acid wash step. COS-7 cells expressing the HA-DAGLα construct were live labelled with the rat anti-HA tag antibody for 30 min at RT, the cells were washed and returned back to the incubator for uptake. After 30 min incubation, the cells were washed in acid buffer and fixed (Figure 6-9c). The cells in Figure 6-9c showed extensive intracellular staining of HA-DAGLα, indicating the internalized antibody-enzyme complexes were left intact after acid wash. However the surface staining was again completely absent, suggesting that the surface bound antibodies were completely stripped at this stage.

The next step was to test if DAGLα can be recycled back to the cell surface. COS-7 cells expressing the HA-DAGLα construct were live labelled with the rat anti-HA tag antibody for 30 min at RT, the cells were washed and returned back to the incubator for 30 min for uptake. After 30 min incubation, the cells were washed in acid buffer (this stage is Figure 6-9c before fixation, surface bound antibodies should have been completely stripped at this stage) and returned back

to the incubator for another 30 min. After this 30 min incubation, surface HA-DAGLα was again detected (Figure 6-9d). These surface antibody-protein complexes detected at step ‘d’ can only come from the intracellular pool of HA-DAGLα because the surface antibody was completely stripped before this incubation. Therefore, the presence of the surface labelling after acid wash and incubation indicates that DAGLα can be recycled back to the cell surface and DAGLα is dynamically trafficking between the cell surface and cytoplasm in COS-7 cells.

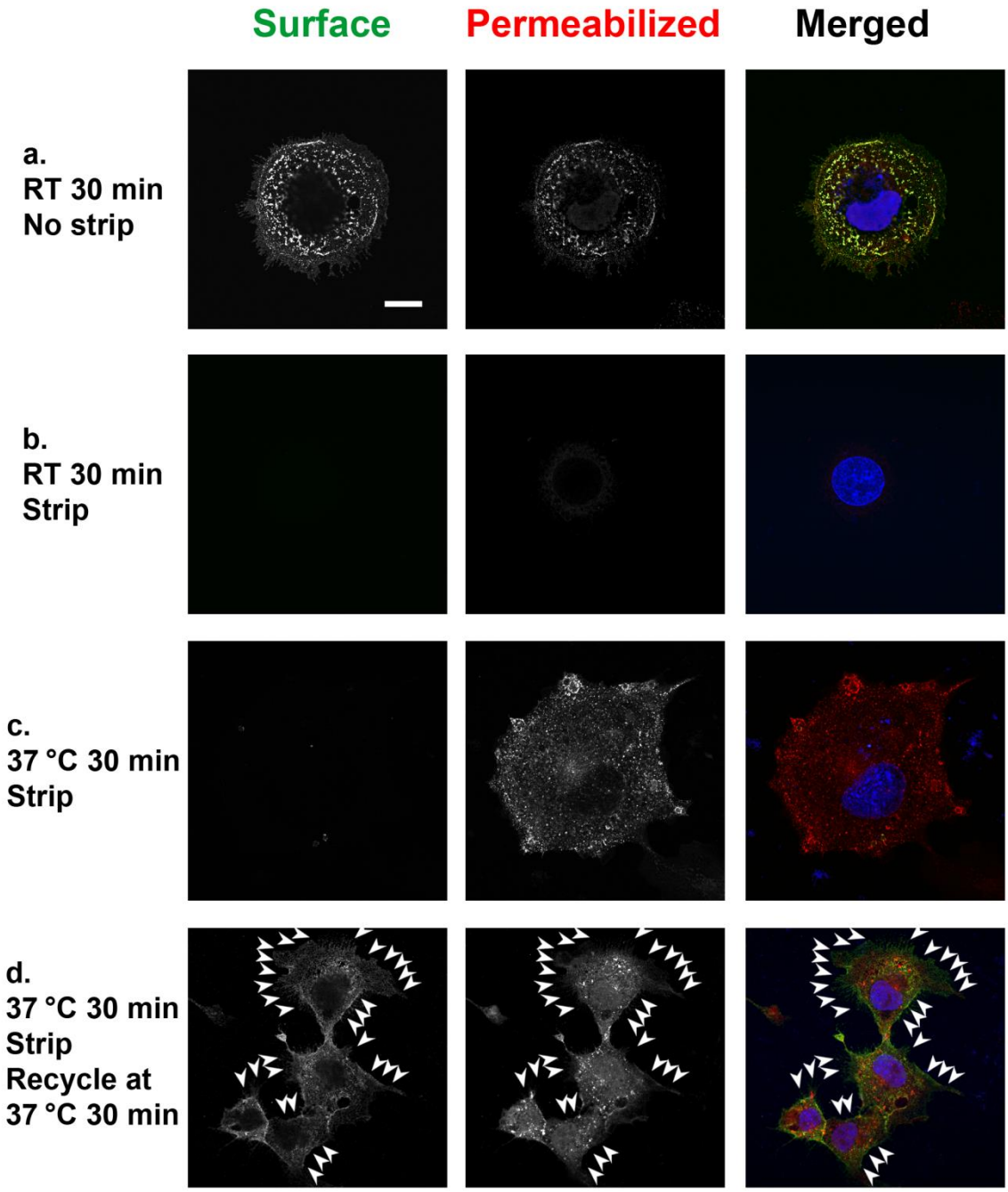

Surface
Permeabilized
Merged
a.
RT 30 min
No strip
b.
RT 30 min
Strip
c.
37 °C 30 min
Strip
d.
37 °C 30 min
Strip
Recycle at
37 °C 30 min

Figure 6-9 DAGLα is recycling in COS-7 cells

COS-7 cells expressing HA-DAGLα were live labelled with the rat anti-HA tag antibody for 30 min at RT. The cells were either washed and fixed directly (a) or washed in a DMEM buffer (pH 3.0-3.5) (b) before fixation. An anti-rat 488 secondary antibody was then used to detect the anti-HA antibody on the cell surface. Results show the cells without the acid wash had extensive labelling for the anti-HA antibody on the cell surface (a). However, after acid wash, the surface staining was completely gone (b), indicating the success of antibody stripping. Another group of COS-7 cells were live labelled and washed as above and returned back to the incubator for uptake for 30 min. After incubation, the cells were washed in acid buffer and either fixed (c) or returned back to the incubator for another 30 min for the internalized antibody-enzyme complexes to recycle before fixation (d). An anti-rat 488 secondary antibody was used to detect the anti-HA antibodies remained on the cell surface. After permeabilization, an anti-rat 594 secondary antibody was used to detect the anti-HA antibodies associated with the internalized HA-DAGLα. Confocal images showing the surface (green stain) and internalized (red stain) HA-DAGLα. As shown by white arrows in (d), surface HA-DAGLα was again detected after antibody stripping and incubation, indicating that the DAGLα can be recycled back to the cell membrane after having been internalized. Scale bar: 20 µm.

6.2.9 DAGLα co-localizes with the early endosome marker, EEA 1 in COS-7 cells

Endocytosis is a process involving many molecules and signalling pathways. The most well-characterized internalization pathway is clathrin-mediated endocytosis (Arancibia-Carcamo et al, 2006). Non-clathrin mediated pathways like caveolin or raft-dependent internalization, macropinocytosos, phagocytosis are less characterised, but these clathrin independent pathways are the key focus of many labs because of their physiological importance (Arancibia-Carcamo et al, 2006; Boucrot et al, 2015; Mayor et al, 2014; Renard et al, 2015; Wu et al, 2014b). Once it has been internalized, the receptor/protein enters the endocytic pathway and will be targeted to distinct endocytic organelles (Arancibia-Carcamo et al, 2006). Previous studies have suggested that both clathrin and caveolin-dependent endocytosis pathways are involved in the early endosomes (Arancibia-Carcamo et al, 2006; Pelkmans et al, 2004). The early endosome, also known as the sorting endosome, is a common sorting station which accepts incoming cargoes internalized from the plasma membrane. It has regions of thin tubular extensions and large vesicles that are located toward the cell periphery (Jovic et al, 2010). The early Endosome Antigen1 (EEA 1) is a well-characterised early endosome marker (Mu et al, 1995) and we wanted to test whether DAGLα co-localizes with this early endosome associated protein. COS-7 cells transfected with HA-DAGLα were antibody fed for 20 min at 4°C, cell were then either fixed at 0 min for a zero time point control or returned back to 37°C incubator for 20 or 60 minutes. Cells were then labelled for surface HA for 0 min control or permeabilized and stained for the anti-HA antibodies that associate with the internalized DAGLα (green channel in (Figure 6-10). The anti-EEA 1 antibody was then used and subsequently labelled by another secondary antibody (shown in red in Figure 6-10). Results of immunostaining are shown in Figure 6-10, co-localization of internalized DAGLα with EEA 1 can be seen at 20 min, but the co-localization is less apparent at 60 min. This is consistent with the notion that the early endosomes accommodate the rapid internalized cargoes, but these cargoes will then be sorted from early endosomes to other organelles like lysosomes for degradation or recycling endosomes for recycling back to the membrane (Arancibia-Carcamo et al, 2006; Jovic et al, 2010). Thus the results suggest that the rapidly internalized DAGLα goes into the early endosomes.

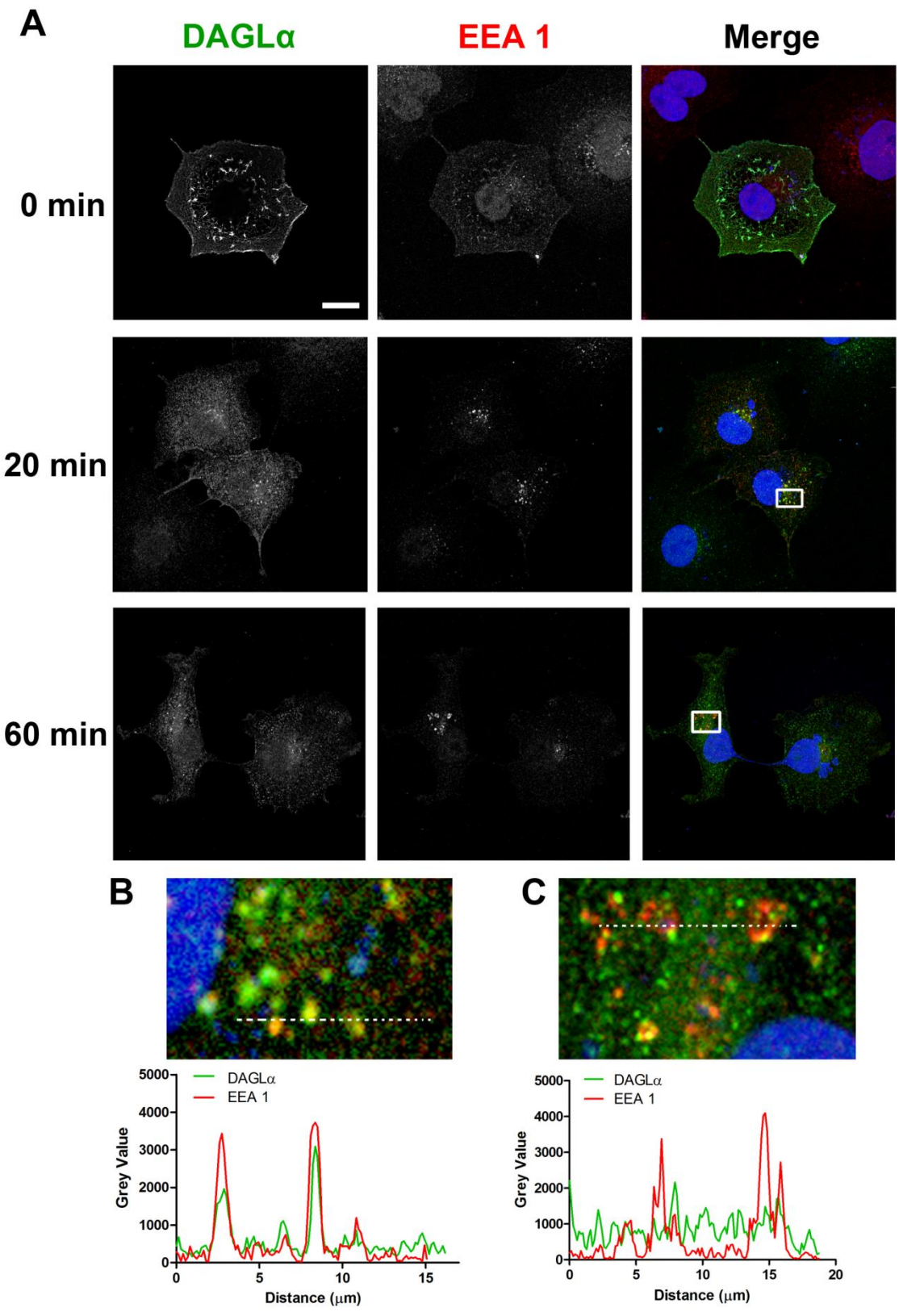

A
DAGLα
EEA 1
Merge
0 min
20 min
60 min
B
C
DAGLα
EEA 1
Grey Value
Distance (μm)
5000
4000
3000
2000
1000
0
0
5
10
15
20

Figure 6-10 DAGLα co-localizes with the early endosome marker, EEA 1 in COS-7 cells

COS-7 cells expressing HA-DAGLα were live labelled with an rat anti-HA antibody for 20 min at 4°C then either fixed at 0 min or return back to 37°C incubator for 20 or 60 minutes. Cells were then labelled for HA tag associated with surface DAGLα for 0 min control or permeabilized and stained for the HA tag that associates with the internalized DAGLα (green channel). Cells were then double immunostained for the early endosome marker EEA 1 (red channel) (A). (B) (C) are zoomed views of the rectangular boxes indicated for 20 and 60 min uptakes respectively. The lower panels of (B) and (C) show the intensity profiles for the indicated dotted lines. The results show the internalized DAGLα co-localize with EEA 1 at 20min, but the co-localization is less apparent at 60 min. Scale bar: 20 μm.

6.2.10 DAGLα co-localizes with the early endosome marker, Rab5 in COS-7 cells

Rab5 is a small GTPase localised to early endosomes and is commonly used as another early endosome marker (Bucci et al, 1992; Majzoub et al, 2015). A previous study reported that the expression of a Rab5 GTPase-deficient mutant resulted in membrane fusion in endocytosis and induced giant endosomes in cells (Stenmark et al, 1994). An anti-Rab5 antibody was used in this study to test if the DAGLα co-localize with this early endosome marker. COS-7 cells expressing HA-DAGLα were used and antibody fed with the rat anti-HA tag antibody for 20 min at 4°C. Cells were then washed and either fixed at 0 min to give a zero time point control or returned back to 37°C incubator for 20 or 60 min before fixation. Cells were then permeabilized and labelled for anti-HA antibodies associated with the internalized DAGLα (shown in green in Figure 6-11). All cells were then double labelled for Rab5 (shown in red in Figure 6-11). As shown in Figure 6-11, similar to EEA 1 co-localization study, co-localization of internalized DAGLα and Rab5 can be observed at 20 min, again the co-localization was less apparent at 60 min. Therefore, we conclude that the internalized DAGLα first goes into the early endosomes and from here it then be sorted into other endocytic organelles.

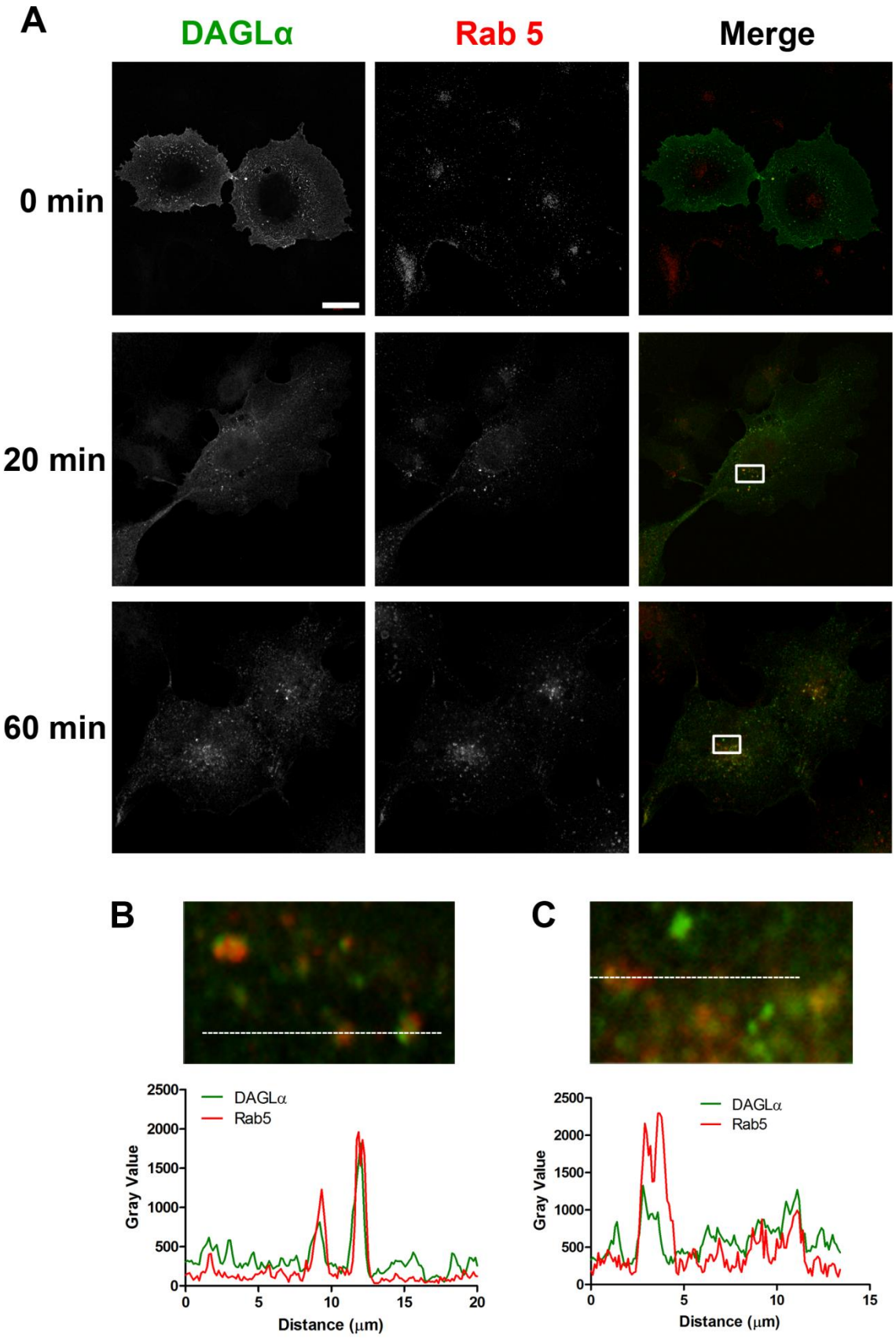

A
DAGLα
Rab 5
Merge
0 min
20 min
60 min
B
C
DAGLα
Rab5
Gray Value
Distance (μm)
2500
2000
1500
1000
500
0
5
10
15
20

Figure 6-11 DAGLα co-localizes with early endosome marker, Rab5 in COS-7 cells

COS-7 cells expressing HA-DAGLα were antibody fed with the rat anti-HA tag antibody for 20 min at 4°C and washed. Cells were then either fixed at 0 min or returned back to the 37°C incubator for 20 or 60 min before fixation. Cells were then labelled for the anti-HA antibodies associated with surface DAGLα for 0 min control or permeabilized and stained for the anti-HA antibodies associated with the internalized DAGLα (green channel). All cells were then double labelled for Rab5 (red channel). Confocal images showing the internalized DAGLα co-localizes with Rab5 at 20 min, but the co-localization is less apparent at 60 min (A). Zoomed views of the rectangular boxes indicated for 20 and 60 min uptakes are shown in (B) and (C) respectively. The lower panels of (B) and (C) show the intensity profiles for the dotted lines. Scale bar: 20 μm.

6.2.11 DAGLα does not co-localize with a lysosome marker, LAMP1 in COS-7 cells

After the receptor/enzyme has been internalized and entered into the early endosomes, the protein cargo can be sorted to late endosomes or recycling endosomes (Arancibia-Carcamo et al, 2006). Late endosomes can mature into lysosomes where the protein is degraded by lysosome proteases (Arancibia-Carcamo et al, 2006). We have shown above that DAGLα is recycling inside of the COS-7 cells. Therefore it's unlikely that the constitutively internalized DAGLα will be sorted into lysosomes for degradation. A lysosome marker, LAMP1 was used to further test our hypothesis. LAMP1 is also called lysosome-associated membrane protein 1, its wild type form is exclusively localized in high density lysosomes (Akasaki et al, 2014). Antibody feeding and internalization assay were performed as shown above, the cells were incubated at 37°C for 40 or 60 min instead of 20 min to allow the endosomes to mature into lysosomes. Cells were double stained for HA epitope tag that associate with the internalized DAGLα (green channel) as well as LAMP1 for lysosomes (red channel). Representative images are shown in Figure 6-12, the results suggest that the internalized DAGLα does not co-localize with LAMP1 at 40 or 60min. Thus we can conclude that DAGLα is mainly recycled back to the plasma membrane after been internalized rather than being degraded in lysosomes.

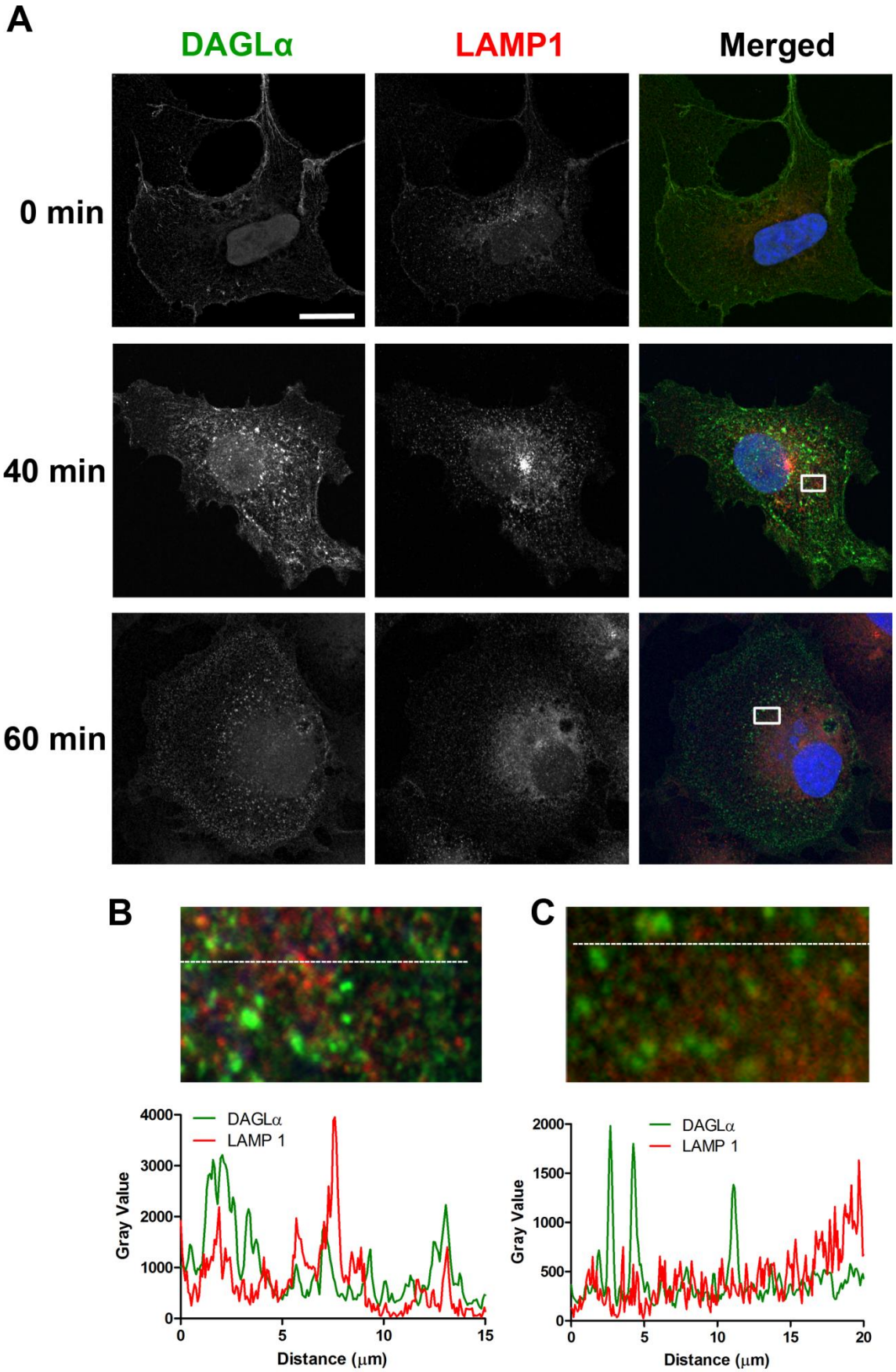

A
DAGLα
LAMP1
Merged
0 min
40 min
60 min
B
C
DAGLα
LAMP 1
Gray Value
Distance (μm)

Figure 6-12 DAGLα does not co-localize with lysosome marker, LAMP1 in COS-7 cells

COS-7 cells were transfected with HA-DAGLα construct, cell were then live labelled with the rat anti-HA tag antibody for 20 min at 4°C. Cells were then either fixed at 0 min or return back to 37°C incubator for 40 or 60 min before fixation. Cells were then permeabilized and double stained with anti-HA antibodies that associate with DAGLα (green channel) and anti-LAMP1 for lysosomes (red channel). Representative confocal images are shown in (A). Zoomed views of the rectangular boxes indicated for 40 and 60 min uptakes were shown in (B) and (C) respectively. The lower panels of (B) and (C) show the intensity profiles for the dotted lines. The results indicate the internalized DAGLα does not co-localize with LAMP1 at 40 or 60 min. Scale bar: 20 μm.

6.2.12 DAGLα does not co-localize with transferrin, a protein internalized by clathrin-mediated endocytosis

Transferrin is a glycoprotein which binds iron tightly and this transferrin-iron complex then binds to the cell surface expressed transferrin receptor which imports the iron into the cells by internalizing the transferrin-iron complex through receptor-mediated endocytosis (Harding et al, 1983; Testa et al, 1991; Tortorella & Karagiannis, 2014). Transferrin receptor-mediated endocytosis plays a crucial role in regulating iron uptake and transport in nearly all eukaryotic cells and since iron is an essential element for cell growth and metabolism, this internalization pathway is tightly regulated inside of the cell (Harding et al, 1983; Testa et al, 1991; Tortorella & Karagiannis, 2014). Transferrin is constitutively internalized in the cell and it is generally regarded that transferrin-iron and transferrin receptor complex enters the cell through clathrin-mediated endocytosis, a internalization pathway that have been extensively studied (Doherty & McMahon, 2009; Liu et al, 2010; Mayle et al, 2012; Pearse & Robinson, 1990). After the transferrin-iron complex has been internalized, iron will be released while transferrin remains bound to the transferrin receptor throughout the endocytic cycle where they are sorted into early endosomes, and subsequently late and recycling endosomes to recycle back to the plasma membrane (Ciechanover et al, 1983; Mayle et al, 2012). We have shown that DAGLα is constitutively internalized and recycled in COS-7 cells, after it has been internalized, DAGLα is also sorted into early endosomes. It might be possible that DAGLα is internalized through clathrin-mediated endocytosis in the same way as transferrin. To test this, a human transferrin conjugated with Alexa Fluor 555 (Tf 555) was used (Czibener et al, 2006). We transfected COS-7 cells with the HA-DAGLα construct, live cells were then used in an antibody feeding and internalization assay. Cells were antibody fed with the anti-rat HA antibody together with Tf 555. Cells were then washed and fixed to get a 0 min control or allowed to internalize for 20 or 40 min before fixation. Cell were then permeabilized and DAGLα detected via the HA tag. Confocal images were taken and representative images are shown in Figure 6-13. Remarkably, the results showed that DAGLα does not co-localize with transferrin in 20 and 40 min internalization. Thus, the results indicate that DAGLα might be associated with a clathrin-independent endocytic pathway.

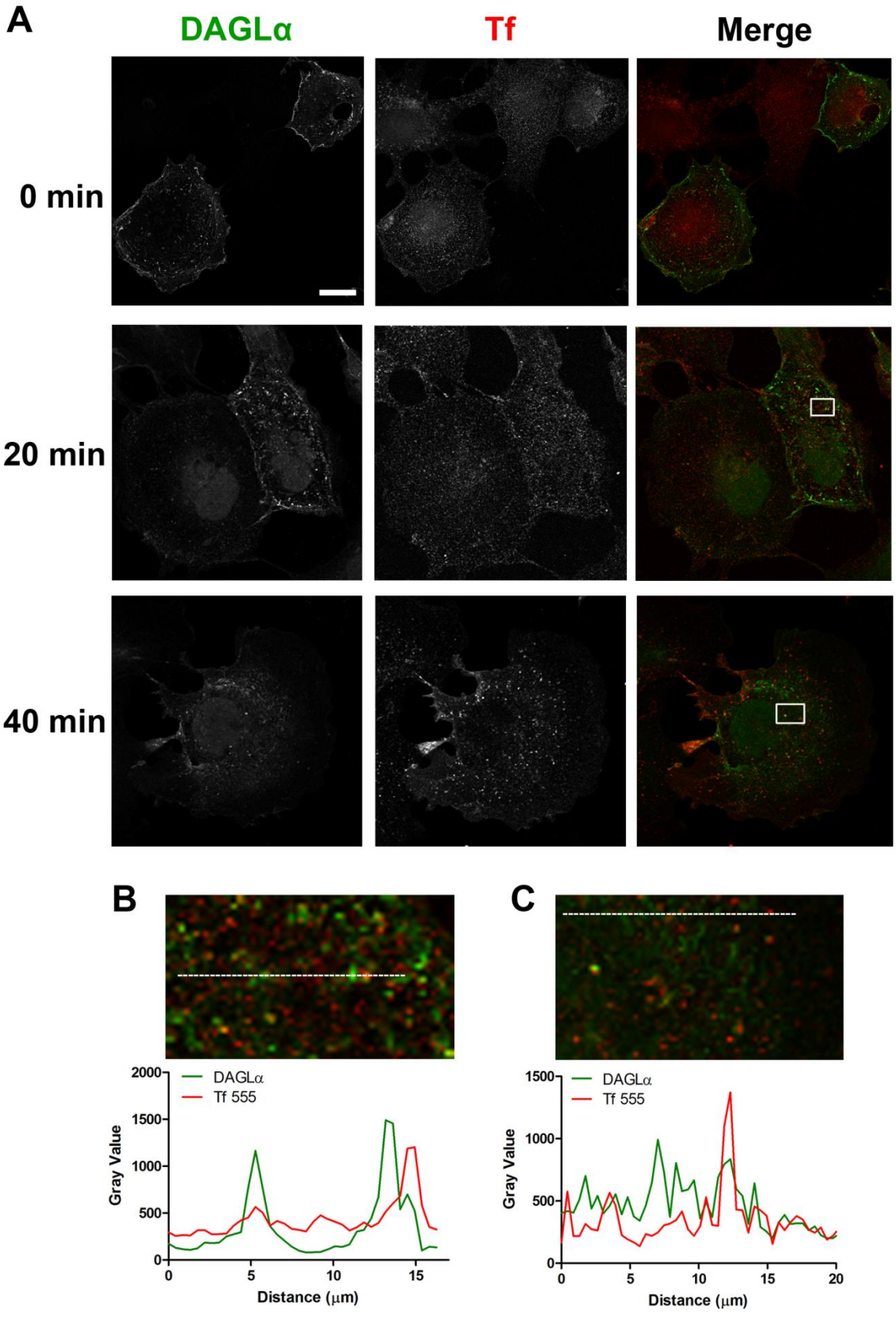

A
DAGLα
Tf
Merge
0 min
20 min
40 min
B
C
DAGLα
Tf 555
Gray Value
Distance (μm)
2000
1500
1000
500
0
0
5
10
15
20

Figure 6-13 DAGLα does not co-localize with transferrin

COS-7 cells expressing HA-DAGLα were live labelled with the anti-HA tag antibody for 20 min at 4°C together with Tf 555. Cells were then either fixed at 0 min or return back to 37°C incubator for various times. Cells were then permeabilized and labelled for HA tag antibody that associates with internalized DAGLα (shown in green). Confocal images were taken and representative images are shown above (A). The zoomed views of the rectangular boxes at 20 min and 40 min are shown in (B) and (C) respectively. The lower panel shows the intensity profiles of the dotted lines in zoomed in images. The results indicate the internalized DAGLα does not co-localize with transferrin at the time points tested. Scale bar: 20 µm.

6.2.13 DAGLα does not co-localize with Caveolin 1, Flotillin 1 and GPI, markers known to be internalized through clathrin independent endocytic pathways

The results above suggest that DAGLα may not internalized by the clathrin-mediated endocytosis pathway. We therefore tested 3 markers that have been shown to be internalized via clathrin independent endocytic pathways to see if DAGLα co-localizes with any of these markers. Caveolae (for 'little caves') are clathrin independent plasma membrane buds that form flask-shaped invaginations on the cell surface (Doherty & McMahon, 2009). This is a relatively well studied clathrin-independent endocytic pathway (Arancibia-Carcamo et al, 2006; Doherty & McMahon, 2009). Caveolin 1 is a protein enriched in caveolae which has been shown to be necessary for caveolar formation (Chaudhary et al, 2014; Doherty & McMahon, 2009; Pelkmans et al, 2004). Flotillin 1 has been shown to be one of the determinants of a second clathrin-independent endocytic pathway in mammalian cells which is necessary for a protein of Cholera Toxin Subunit B (CTxB) uptake (Doherty & McMahon, 2009; Glebov et al, 2006). Glycosylphosphatidylinositol (GPI)-linked proteins have also been shown to be involved in clathrin-independent endocytic pathways (Ait-Slimane et al, 2009; Sabharanjak et al, 2002). A Caveolin 1-GFP (Cav 1-GFP) construct, a polyclonal Flotillin 1 antibody and a GPI-GFP construct were used for co-localization study (all reagents are gifts from Dr. Oleg O. Glebov, King's College London) (Glebov et al, 2006; Glebov & Nichols, 2004). COS-7 cells were co-transfected with HA-DAGLα and Cav 1-GFP or GPI-GFP or HA-DAGLα only for Flotillin 1 double staining. Cells were live labelled with the anti-HA tag antibody for 20 min at 4°C and washed. Cells were then either fixed at 0 min or return back to 37°C incubator for 20 min incubation before fixation. Cells were then permeabilized and labelled for HA tag that associates with the internalized DAGLα. Cells expressing HA-DAGLα only were then double stained for Flotillin 1. Confocal images were taken and representative images and intensity profiles were shown in Figure 6-14. The results suggest that DAGLα does not co-localize with any of these markers, indicating that DAGLα may not internalize through the Caveolin 1, Flotillin 1 or GPI-linked protein dependent endocytic pathways.

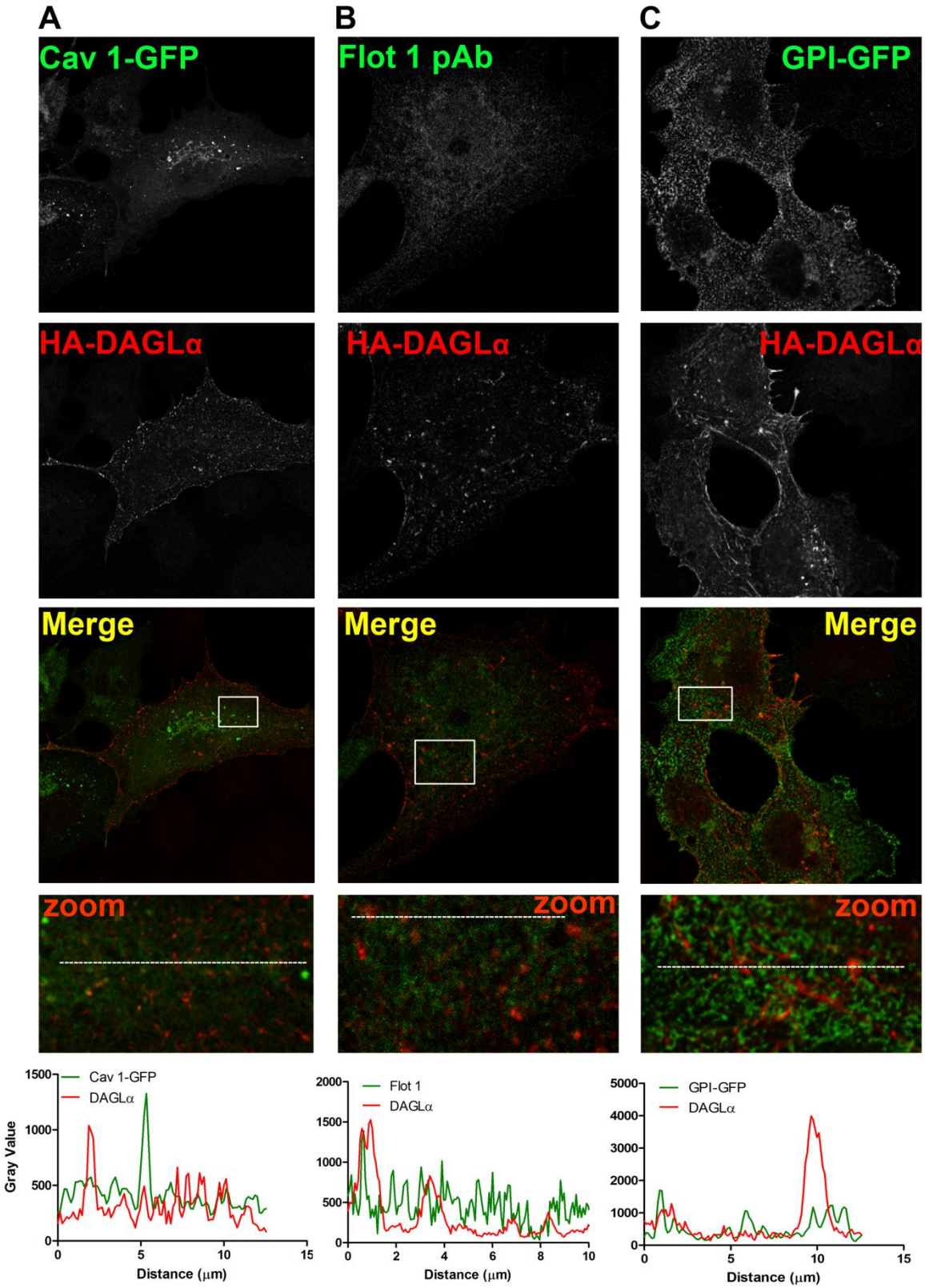

A
B
C
Cav 1-GFP
Flot 1 pAb
GPI-GFP
HA-DAGLα
HA-DAGLα
HA-DAGLα
Merge
Merge
Merge
zoom
zoom
zoom
Gray Value
Cav 1-GFP
DAGLα
Distance (μm)
Flot 1
DAGLα
Distance (μm)
GPI-GFP
DAGLα
Distance (μm)

Figure 6-14 DAGLα does not co-localize with Caveolin 1, Flotillin 1 and GPI, markers known to be internalized through clathrin independent endocytosis pathways

COS-7 cells were co-transfected with HA-DAGLα and Cav 1-GFP or GPI-GFP or HA-DAGLα only for Flotillin 1 double staining. Cells were live labelled with the anti-HA tag antibody for 20 min at 4°C and washed. Cells were then either fixed at 0 min or return back to 37°C incubator for 20 min incubation before fixation. Cells were then permeabilized and labelled for HA tag that associates with internalized DAGLα (shown in red). Cells expressing HA-DAGLα only were then double immunostained for Flotillin 1. Confocal images were taken and representative images were shown above (A-C). The bottom panel shows the intensity profiles for the dotted lines in zoomed views. The results suggest that DAGLα does not co-localize with any of these three markers. Scale bar: 20 µm.

6.2.14 Ionomycin has an effect on DAGLα internalization in HEK 293 cells

Having established that DAGLα can be cycled to and from the cell surface, I next wanted to determine how this might be regulated. Calcium stimulated 2-AG synthesis has been reported in various systems like cells lines, neuron cultures and tissues (Bisogno et al, 2003; Bisogno et al, 1999b; Hashimotodani et al, 2007; Kondo et al, 1998; Stella et al, 1997). For example, treatment of COS-7 cells with the calcium ionophore ionomycin significantly increased the amount of 2-AG that the cells produce and release in a DAGL dependent manner (Bisogno et al, 2003). Also calcium influx in the post-synaptic neuron was able to increase 2-AG signalling at the synapse (Hashimotodani et al, 2007). DAGLα can also directly interact with CamKII (Shonesy et al, 2013). As one of the main enzyme responsible for 2-AG synthesis, the localization of DAGLα might affect the production and release of 2-AG and calcium influx into the cell might in turn affect the distribution of DAGLα on plasma membrane or cytosol. Therefore, we tested the effects of ionomycin on DAGLα internalization and trafficking in COS-7 cells.

COS-7 cells expressing HA-DAGLα were antibody fed with the rat anti-HA antibody at 4°C and washed, 2 μM ionomycin or equivalent DMSO vehicle control were then added into cell medium and cells were returned back to incubator for various times to allow internalization. The results pooled from four experiments showed no significant difference at 20 or 40 min, however a significant effect at 60 min treatment was observed (Figure 6-15) with less internalised receptor seen at this time point after treatment with ionomycin. One possible explanation might be the influx of calcium speeds up the trafficking of DAGLα inside of the cell, therefore more DAGLα protein is recycled back to the cell membrane after 60 min treatment. Indeed, a previous study has shown that ionomycin treatment increased the amount of 2-AG released into the cell medium (Bisogno et al, 2003). However, the rate of internalization was not significantly changed at earlier time points (20 and 40 min) and this does not support this hypothesis. Another explanation might be enzyme degradation. As shown above in Figure 6-6A, long term treatment of HEK293 cells with CB1 agonist reduced the CB1 receptor internalization rate back to control level at 100 min, it has been reported that the CB receptor degradation happens after long term exposure to the agonists (Hsieh et al, 1999). Therefore it might be possible the long term treatment of ionomycin may have caused degradation of DAGLα. However, further study will be required to confirm the effects of calcium on DAGLα internalization and trafficking.

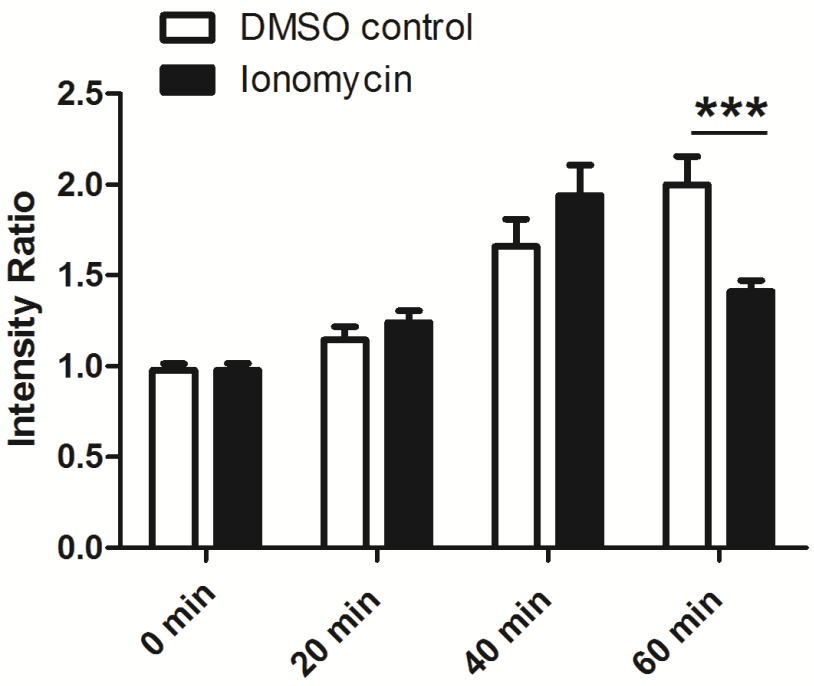


Figure 6-15 Ionomycin has an effect on DAGLα internalization in COS-7 cells

COS-7 cells expressing HA-DAGLα were live fed with the rat anti-HA tag antibody for 20 min at 4°C and washed. Cells were then either fixed at 0 min or returned back to 37°C incubator for 20, 40 or 60 min in 2 μM Ionomycin medium or 0.2% DMSO control. An anti-rat 488 conjugated secondary antibody was used to detect HA-DAGLα remaining on the cell surface. Cells were then permeabilized and stained with an anti-HA tag antibody associates with the internalized DAGLα. The ratio of internalized and surface DAGLα intensity was then calculated. Using Ionomycin to raise the intracellular level of calcium has a significant effect on DAGLα internalization at 60 min treatment. Each bar represents the mean ± SEM (n=4, 10-15 fields were randomly taken from each coverslip, ~100 cells quantified for each group)); *<0.05; ** P < 0.01; *** P < 0.001. One-way ANOVA followed by Bonferroni post-tests.

6.2.15 Peptide 1 does not have significant effect on DAGLα internalization

AP (adaptor protein) complexes are a class of proteins that connect cargo molecules with the components of the vesicle coat during endocytosis, and AP-2 is one of the most important and most studied adaptors in clathrin-dependent endocytosis (Farsad et al, 2003; Heilker et al, 1996; Traub, 2003; Traub, 2009). It mediates the formation of clathrin-coated vesicles from the plasma membrane for endocytosis by providing a link between clathrin and the receptor complex (Traub, 2003; Traub, 2009). AP-2 also plays an important role in cargo selection, making it a core sorting adaptor during endocytosis (Traub, 2009). An AP-2 binding motif (KTHLRRRSSQLK) that is conserved within the intracellular domains of all GABA(A) receptor beta subunit isoforms has been identified to be important in regulating the number of GABA(A) receptors available at the cell surface (Kittler et al, 2005). The phosphorylation of serine in this motif inhibits the AP-2 binding and therefore inhibits the GABA(A) receptor internalization (Kittler et al, 2005). Furthermore, when introduced into cells a dephosphorylated peptide mimetic of the AP-2 binding motif enhanced the amplitude of miniature inhibitory synaptic current and whole cell GABA(A) receptor current, indicating the inhibition of GABA(A) receptor endocytosis after peptide treatment (Kittler et al, 2005). A bioinformatics study revealed that the N-terminal of DAGLα contains a similar motif (RRRWS) which is conserved across species (human, mouse and fly) but not conserved in DAGLβ (Gareth Williams, unpublished data). Therefore we postulated that this possible AP-2 binding motif might play a role on DAGLα endocytosis. A peptide named “peptide 1” corresponding to the N-terminal of DAGLα which contains the motif was synthesised in tandem with a well characterised peptide that transports cargos into cells (Bechara & Sagan, 2013; Derossi et al, 1994; Thoren et al, 2000). We treated the HA-DAGLα expressing COS-7 cells with 100 ng/ml peptide 1 or a control peptide for 1 h before antibody feeding. After antibody feeding, cells were allowed to internalize with the same concentration of peptide 1 or the control peptide in the incubator for various times. Cells were then fixed and a 488 conjugated secondary antibody was used to detect the surface DAGLα pool. Cells were then permeabilized and a 594 conjugated secondary antibody was used to detect the internalized DAGLα pool. The intensity ratio of the internalized and surface DAGLα was then calculated. The results are shown in Figure 6-16, although there was a trend of a reduced internalization rate after peptide 1 treatment at 20 or 40 min internalization, these effects were not statistically significant. Therefore the AP-2 like binding motif in DAGLα may not be responsible for the enzyme internalization. These results together with

the transferrin co-localization study showed above suggest that DAGLα may not internalized by the typical clathrin-mediated endocytosis pathway.

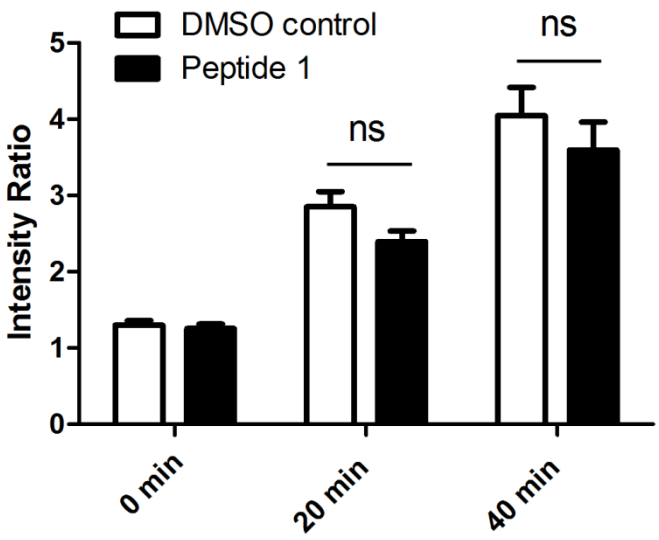


Figure 6-16 Peptide 1 does not have significant effect on DAGLα internalization

COS-7 cells expressing HA-DAGLα were pre-treated with 100 ng/ml peptide 1 (MPGIVVFRRRWSVGSDDRQIKIWFQNRRMKWKK-NH2; sequence shown in red is corresponding to the N-terminal of DAGLα which contains the putative AP-2 binding motif; sequence in green is the Antennapedia Peptide which enables a wide range of peptides to cross cellular membranes (Bechara & Sagan, 2013; Derossi et al, 1994; Thoren et al, 2000) or a control peptide for 1 h in cell culture medium. Cells were then live labelled with the rat anti-HA tag antibody for 20 min at 4°C and washed. Cells were then either fixed at 0 min or return back to 37°C incubator for 20 or 40 minutes with the same concentration of peptide 1 or control peptide in cell medium before fixation. An anti-rat 488 secondary antibody was used to detect HA tag associate with surface DAGLα. Cells were then permeabilized and an anti-rat 594 secondary antibody was used to detect HA tag associated with the internalized DAGLα pool. The ratio of internalized / surface DAGLα intensity was then calculated. Peptide 1 has a trend to reduce DAGLα internalization, but this effect does not reach statistical significance. Each bar represents the mean ± SEM (n=3, 10-15 fields were randomly taken from each coverslip, ~100 cells quantified for each group); *<0.05; ** P < 0.01; *** P < 0.001. One-way ANOVA followed by Bonferroni post-tests.

6.2.16 Mutation of P975L in DAGLα does not stop DAGLα from being expressed on the cell surface in COS-7 cells

We have reported above that DAGLα co-localizes with Homer in hippocampal neurons. This result was supported by a bioinformatics study that revealed the C-terminal tail of human (and rodent) DAGLα contains a consensus PPxxF Homer-binding motif (Jung et al, 2007). Co-immunoprecipitation of Homer 2 and DAGLα protein has also been reported (Jung et al, 2007). Moreover, two point mutations in the Homer binding motif in DAGLα (P975L and F978R) abolished Homer and DAGLα co-immunoprecipitation, suggesting that these two proteins interact selectively through the PPxxF motif (Jung et al, 2007). Furthermore, the authors also reported that this P975L mutation changed the localization of DAGLα from the plasma membrane to the cytosol in Neuro-2a cells, suggesting that the interaction between Homer and DAGLα is required for the cell surface localization of DAGLα (Jung et al, 2007). It was of interest for us to test if this P975L mutation can stop the DAGLα from being expressed on the cell surface. COS-7 cells were transfected with either HA-DAGLα or a HA-DAGLα-P975L mutant construct, cells were then antibody fed and allowed to internalize for various time. Results, shown in Figure 6-17, clearly reveal green staining for the surface expressed DAGLα and red staining for the internalized DAGLα. The results clearly show that the P975L mutation in DAGLα did not stop DAGLα from being expressed on the cell surface. Also visual inspection did not indicate any obvious change in the rate of internalization of the mutated enzyme. Therefore, we can conclude that this Homer binding motif in DAGLα is not responsible for surface localization of DAGLα in COS-7 cells. Further study will be needed to test whether this mutant changes the localization of DAGLα in neuronal cell lines or neurons.

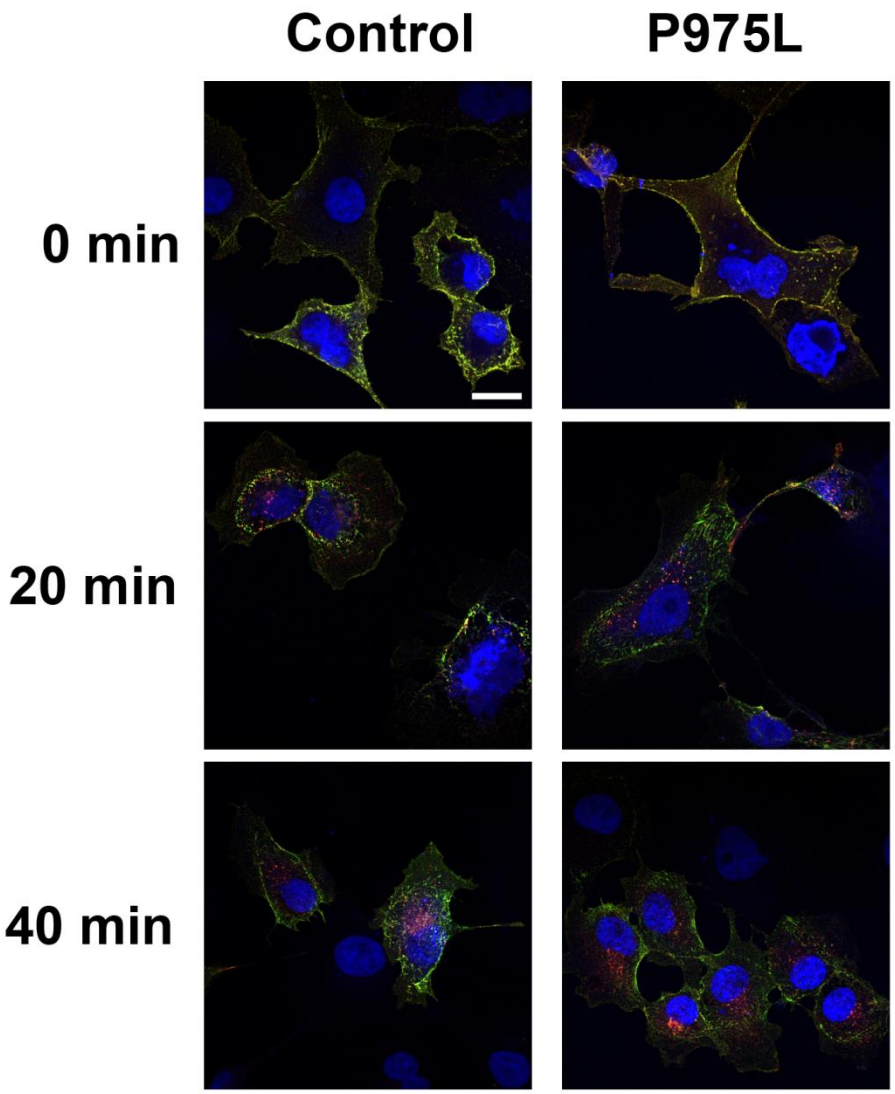


Figure 6-17 Mutation of P975L in DAGLα does not stop DAGLα from being expressed on the cell surface in COS-7 cells

COS-7 cells expressing HA-DAGLα or HA-DAGLα-P975L mutant (P975 is a site within a potential homer binding motif in DAGLα) were live labelled with the rat anti-HA tag antibody for 20 min at 4°C and washed. Cells were then either fixed at 0 min or returned back to 37°C incubator for various times in cell culture medium before fixation. An anti-rat 488 antibody was used to detect HA tag associated with surface DAGLα (shown in green). Cells were then permeabilized and an anti-594 antibody was used to stain the internalized DAGLα pool (shown in red). Confocal images showing merged images of surface DAGLα (green) and DAGLα after permeabilization (red). Both control and the P975L mutant expressing cells have DAGLα expressed on the cell surface at different time points in COS-7 cells. Scale bar: 20 µm.

6.2.17 DAGLα is constitutively internalized in rat hippocampal neurons

We have shown above that DAGLα is constitutively internalized and sorted to early endosomes and is then recycled back to the plasma membrane in COS-7 cells. However, our ultimate goal is to study DAGLα in dendritic spines in neurons, where the enzyme modulates retrograde signalling and synaptic activity. Therefore, we tested if DAGLα can be internalized in hippocampal neurons. Rat hippocampal neurons were cultured from E18.5 rats. Neurons were transfected with HA-DAGLα at 10 DIV and 3 days later, live cells were antibody fed with the rat anti-HA antibody at RT and washed. Cells were then returned back to the incubator for 20 min to allow endocytosis. Neurons were then fixed and an anti-rat 488 antibody was used to detect HA tag associate with surface DAGLα (shown in green in Figure 6-18). Cells were then permeabilized and an anti-594 antibody was used to stain the internalized DAGLα pool (shown in red in Figure 6-18). As shown in Figure 6-18, internalized DAGLα was detected in neuronal dendrites (white arrows in Figure 6-18). Thus we can conclude that DAGLα is constitutively internalized at the dendrites in hippocampal neurons.

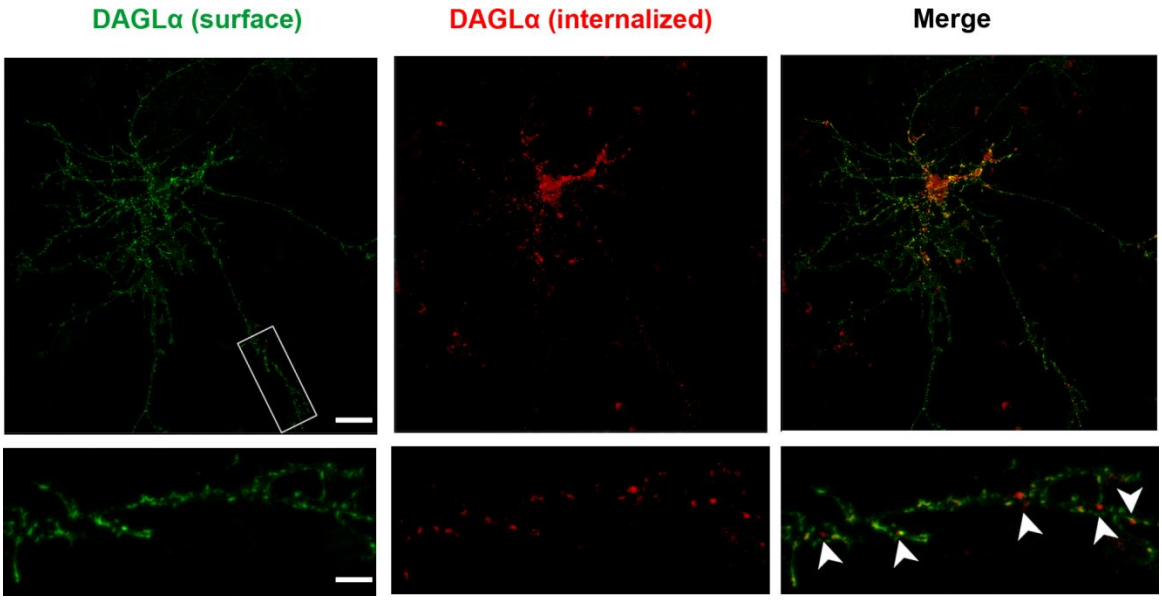


Figure 6-18 DAGLα is constitutively internalized in rat hippocampal neurons

Cultured rat hippocampal neurons were transfected by HA-DAGLα at 10 DIV. 3 days later, neurons were live labelled with the rat anti-HA tag antibody for 20 min at RT and washed. Cells were then returned back to 37°C incubator for 20 min in cell culture medium. Neurons were then fixed and an anti-rat 488 antibody was used to detect HA tag associate with surface DAGLα (shown in green). Cells were then permeabilized and an anti-594 antibody was used to stain the internalized DAGLα pool (shown in red). Lower panel shows the zoomed views of the rectangular area. White arrows indicate the internalized DAGLα at the dendrites. These results suggest DAGLα can be internalized at the dendrites of rat hippocampal neurons after 20 min internalization. Scale bar: 20 µm and 5 µm.

Chapter 7 Discussion

7.1 Why study the role of eCB, FGFR and TrkB signalling on neuroblast migration in the RMS?

In the introduction to this thesis I have highlighted some of the key requirements for the development and functioning of the brain, and commented on the role that the eCB signalling system plays in axonal growth and guidance, neurogenesis and synaptic plasticity. From an experimental perspective, the first study that I undertook was to determine the role that DAGL-dependent eCB signalling plays in neuroblast migration in the postnatal brain. In this context the SVZ has recently emerged as a crucial neurogenic niche. Thousands of neural precursors are created in the SVZ of postnatal and adult mammalian brains and migrate in chains via the RMS to the OB to differentiate into interneurons (Doetsch & Alvarez-Buylla, 1996; Ming & Song, 2005; Ming & Song, 2011). This tangential migration of neuroblasts in the RMS is highly characteristic with overall movement directed towards the OB. However, directed movement is not absolute with other studies demonstrating that at any one time neuroblasts can be found moving back towards the SVZ as well as moving toward the OB (Bagley & Belluscio, 2010; Nam et al, 2007). Removal of the OB has no or little effect on guidance, suggesting that this is mainly determined by factors emanating from the SVZ and/or synthesised locally within the stream (Bagley & Belluscio, 2010). Studies on the migration along the RMS in the recent years have become a useful system in revealing the potential molecular mechanisms involved in cell migration (Ming & Song, 2005; Ming & Song, 2011). Many proteins and signalling pathways, including several already to be known involved in cancer cell migration, such as adhesion molecules (PSA-NCAM, integrin), extracellular cues (NRGs, Slits), GABA, Ephrins and neurotrophins have been found to contribute to neuroblast migration in the RMS (Anton et al, 2004; Bolteus & Bordey, 2004; Cavallaro & Christofori, 2004; Chazal et al, 2000; Chiaramello et al, 2007; Mobley & McCarty, 2011; Nguyen-Ba-Charvet et al, 2004), but the whole system is far from being fully understood (Ming & Song, 2011). Some studies have also provided evidence that the progenitor cells produced by the SVZ can migrate out of the RMS to the site of brain injury to contribute to brain repair (Arvidsson et al, 2002; Christie & Turnley, 2012; Gotts & Chesselet, 2005a; Young et al, 2011). It is therefore fundamental to fully understand how the migration is regulated in the RMS for potential therapeutic applications.

The eCB system consists of several endogenous lipids, their target CB1 and CB2 receptors and enzymes responsible for their synthesis and degradation. CB1 and CB2 receptors are G-protein coupled receptors (GPCRs), they are found to be expressed in the developing and adult brain including neural stem cells and their progeny (Zhou et al, 2014). The CB1 receptor is the most abundant GPCR in the brain and is activated by endogenous lipids, predominantly 2-AG that can be synthesised on demand by the DAGLs (Ben Amar, 2006; Bisogno et al, 2003; Matsuda et al, 1990; Mechoulam et al, 1995; Piomelli, 2003; Sugiura et al, 1995; Zhou et al, 2014). The eCB system regulates many physiological processes in the CNS including neurogenesis, axon guidance and synaptic plasticity (Harkany et al, 2008; Oudin et al, 2011b; Zhou et al, 2014). Recent studies have highlighted additional important roles for eCB signalling in regulating proliferation of neural progenitors in both the adult hippocampus and SVZ and regulating axonal growth and guidance, making it a key signalling system for the complex architecture and efficient wiring of the CNS (Gao et al, 2010; Goncalves et al, 2008; Oudin et al, 2011a; Zhou et al, 2015). Indeed, knock-out mice studies together with pharmacological perturbation *in vivo* indicate that the eCB signalling controls migration both pre- and post-natally, regulating interneuron positioning in the developing cortex and hippocampus (Berghuis et al, 2005; Berghuis et al, 2007; Mulder et al, 2008). A more recent study from our lab has suggested that the eCB system regulates the motility of stem cell-derived neuroblasts in the RMS, but this was mainly based on *in vitro* explant cultures where the polarity of the stream is lost (Oudin et al, 2011a). Therefore it was deemed to be of interest to look in considerable detail at the role of the eCB signalling on the neuroblast migration in relatively intact RMS.

For reasons that will be discussed in more detail later, the second focus was to test the effects of inhibiting FGFR function on migration. The FGFR signalling system is relatively complex with 4 receptors (FGFR1-4) and a multitude of “conventional” growth factor ligands. FGFR1-4 are highly conserved transmembrane tyrosine kinase receptors with each receptor containing an extracellular domain composed of three immunoglobulin (Ig) domains, a single transmembrane domain, and an intracellular tyrosine kinase domain. Alternative mRNA splicing of the extracellular domains has been reported in FGFRs and the resultant isoforms of these receptors have different ligand binding properties. Also these alternative spliced receptor variants together with their ligands, the FGFs, are expressed in specific spatial and temporal patterns thus allowing control of the specificity of FGF-FGFR interaction (Burke et al, 1998; Ornitz et al, 1996; Turner &

Grose, 2010). FGFs are secreted glycoproteins that are usually released by heparinises, proteases or specific FGF binding proteins. 18 FGFs has been found so far and most FGFs activate a particular subset of FGFRs (Burke et al, 1998; Reuss & von Bohlen und Halbach, 2003; Turner & Grose, 2010; Yang et al, 2015). This highly complex signalling system regulates a multitude of physiological functions and is involved in developmental and repair processes in almost all mammalian tissues (Turner & Grose, 2010). All four FGFRs have been found to be expressed in the developing and adult brain. Among these receptors, FGFR1 is expressed mainly on neuronal population, while FGFR2 and FGFR3 are mainly expressed by glia and other cell types (Zechel et al, 2010). FGF-2 has been reported to be the most abundant FGF in the CNS and is a high affinity ligand for the FGFR1 suggesting widespread functional coupling between this ligand and receptor pair (Zechel et al, 2010). Previous studies have shown that the FGFR signalling plays a key role in cell migration and this signalling pathway is implicated in the metastasis of many types of cancer (Attia et al, 2015; Bobbs et al, 2012; Cao et al, 2013b; Kadam et al, 2012; Nakamura et al, 2001; Turner & Grose, 2010). Molecules like selective FGFR inhibitors, specific FGFR blocking antibodies, FGF ligand traps or recombinant FGFs have therefore been developed for potential therapeutic applications by many pharmaceutical companies (Turner & Grose, 2010). However, further understanding of FGFR signalling on cell migration in the postnatal brain is still needed to better facilitate translational work.

As alluded to earlier, our motivation for studying the role of the FGFR in neuroblast migration in the RMS was largely driven by previous reports of direct cross-talk between the FGFR and the eCB system in neurons (Williams et al, 2003). This study provided evidence for FGFR dependent activation of PLCγ, leading to the synthesis of substrate for the DAGLs and resulting in DAGL-dependent activation of the CB1 receptor in a pathway that promotes axonal growth (Williams et al, 2003). This FGFR-eCB pathway can also be directly activated by cell adhesion molecules (CAMs), like NCAM, binding to the FGFR and NCAM has also been reported to be important for neuroblast migration in the RMS (Battista & Rutishauser, 2010; Cremer et al, 1994; Doherty & Walsh, 1996; Schmid & Maness, 2008; Williams et al, 1994a; Williams et al, 2001). Thus there is a clear basis for investigating whether this CAM-FGFR-eCB signalling cascade might play a role in the regulation of neuroblast migration in the RMS. We reasoned that a detailed comparison of the roles played by the FGF and eCB receptors in the regulation of neuroblast migration in the

RMS could serve as a critical way to test for direct cross-talk between these pathways in this system.

At the outset of the study we also appreciated that we might need to consider interactions between other growth factors and the eCB system. In this context BDNF is one of the neurotrophins that helps to support the survival of existing neurons, and promotes the growth and differentiation of new neurons. TrkB is the main receptor for BDNF and it interacts with BDNF in a ligand-specific manner (Acheson et al, 1995; Huang & Reichardt, 2001; Yamada & Nabeshima, 2003). BDNF signalling has been shown to be important for the SVZ neurogenesis of the mammalian brain (Bath et al, 2012). Previous studies have also shown that BDNF and TrkB are expressed throughout the SVZ-OB system and the TrkB receptor is expressed by the migrating neuroblasts. Additionally, BDNF has a motogenic effect on neuroblasts in RMS explant cultures (Bath et al, 2008; Chiaramello et al, 2007; Snapyan et al, 2009), indicating an important role for BDNF and TrkB in the RMS. However, the role of BDNF signalling in regulating neuroblast motility and directionality along the entire intact stream is less clear as an antibody that can block BDNF function has been reported to increase neuroblast motility within the RMS without disrupting guidance (Bagley & Belluscio, 2010), while another report showed that inhibiting BDNF signalling in the adult mouse brain with a TrkB-Fc impairs RMS neuroblast migration and directionality (Snapyan et al, 2009). Thus it was interesting to re-investigate the role of BDNF signalling on neuroblast motility and guidance along different regions of the stream.

The direct cross-talk between the BDNF and eCB signalling has already been reported (Aso et al, 2008; Berghuis et al, 2005; Blazquez et al, 2015; Lemtiri-Chlieh & Levine, 2010; Maison et al, 2009). Interestingly, a wide range of mechanisms can account for this cross-talk. For example, BDNF has been shown to be able to increase the expression of CB1 receptors by increasing gene transcription and thereby increasing neuronal sensitivity to 2-AG in cultured cerebellar granule neurons (CGNs) (Maison et al, 2009). A electrophysiology study has revealed that eCB release at inhibitory synapses in the neocortex can be triggered by BDNF-TrkB signalling pathway (Lemtiri-Chlieh & Levine, 2010). Local administration of BDNF, but not other neurotrophins (NGF, NT-3), in the hippocampus was able to reverse the anxiety-like phenotype seen in CB1 knock-out mice (Aso et al, 2008) and injection of a viral vector expressing the CB1 receptor into the dorsolateral striatum of R6/2 mice, a well-established model of Huntington's disease (HD), rescued the expression of BDNF as well as the HD-like molecular-pathology markers in these

animals, supporting the important role of CB1/BDNF axis in disease (Blazquez et al, 2015). There is also evidence that activation of the CB1 receptor can result in a ligand independent transactivation of the TrkB receptor in a PC12 cell line (Berghuis et al, 2005). In this study a Boyden chamber assay showed that a CB1 agonist stimulated interneuron migration, and that this could be blocked by K252a, a TrkB inhibitor, indicating that the eCBs can use TrkB receptor-dependent signalling pathways to regulate interneuron migration (Berghuis et al, 2005). Previous work in our lab has shown that activation of FGFR signalling can lead to activation of PLCγ to synthesize DAG, with DAGL then using this substrate to generate 2-AG to activate the CB1 receptor. This direct cross-talk between the FGFR and the CB1 receptor promotes axonal growth, a biological response that shares many features with cell migration (Williams et al, 2003). It is also possible that activation of TrkB signalling might lead to direct cross-talk with eCB signalling via a PLC dependent mechanism. Indeed, such a mechanism has recently been reported to be accountable for BDNF interactions with eCBs in the regulation of cocaine-induced synaptic plasticity in mouse dopaminergic neurons (Zhong et al, 2015). For all of the above reasons it was clearly of interest to compare the involvement of the eCB, FGFR and TrkB signalling pathways in neuroblast migration within the RMS.

7.2 eCB signalling on neuroblast migration in the RMS

In the present study we have used time-lapse imaging on acute brain slices to determine if and how eCB signalling regulates migration in the RMS within intact brain slices. To achieve this, *in vivo* postnatal electroporation and subsequent acute brain slice preparation followed by time-lapse imaging was used. Postnatal electroporation can efficiently transfect SVZ progenitor cells, which in turn generate neuroblasts migrating along the RMS. Here we used a GFP-expressing plasmid for electroporation to label the neuroblasts migrating along the RMS. Acute brain slice cultures make pharmacological treatment possible and there are excellent small molecules available for the targeting of the eCB system. Using confocal spinning disk time-lapse microscopy on acute brain slice cultures, neuroblast migration can be monitored in an environment closely resembling the *in vivo* condition. Moreover, neuroblast motility can be tracked and quantitatively analyzed (Sonego et al, 2013b). In this part of study we have used live cell imaging of neuroblasts in intact brain slices to determine if eCB signalling has roles beyond their motogenic effects on neuroblast migration - previously documented with explant cultures (Oudin et al, 2011a). Our

results show that the eCB system can regulate both neuroblast motility and local guidance throughout the stream.

As discussed above, many molecules can regulate neuroblast migration in the RMS, but most of these have been shown to primarily affect motility rather than regional guidance, and examples include GABA (Bolteus & Bordey, 2004) and metalloproteases (Bovetti et al, 2007). The eCB system has also been shown to have largely motogenic effects on cultured cells. Activation of CB receptors promotes neuroblast migration from explants cultures, while inhibitions of eCB signalling reduce this (Oudin et al, 2011a). Both CB1 and CB2 antagonists have been shown to have similar effect on altering neuroblast morphology within the RMS *in vivo* and inhibiting the migration of RMS neuroblasts in cultured explants *in vitro* (Oudin et al, 2011a). In this context, we first inhibited eCB signalling by using CB1 and CB2 antagonists together because both have previously been shown to contribute to the inhibition of eCB signalling and we wanted to fully inhibit the pathway.

In this study, treating the cultured brain slices with CB1/CB2 antagonists resulted in the cells spending longer periods of time in an immobile state (from ~25% in control to ~45% in treated). These results are consistent with the *in vitro* explant culture studies where nucleokinesis (movement of the cell body and nucleus, which is key for directed neuronal migration) of the neuroblast was reduced approximately threefold per hour relative to the control when cells were treated with CB1 or CB2 antagonists (Oudin et al, 2011a). Interestingly, the average speed of migration was not significantly altered when treating with CB1/2 antagonists. Thus, we found the primary effect of the eCB on motility is on the length of time the neuroblasts are immobile, rather than on the speed of movement when they do migrate. One possibility is that a maintained momentum might help propel cells towards the OB. If the cells for some reason become immobile during their travel, they might lose their sense of direction and have to re-explore their immediate environment, thus the momentum is lost and cells spend more time exploring their local environment.

The CB1/CB2 antagonist treatments not only resulted in the neuroblast migrating over shorter distances towards the OB compared to vehicle treated control cells, but also the net migration towards the OB and the total cell displacement were significantly reduced. In addition, control cells often display a predominant unipolar morphology usually oriented towards the OB, while cells treated with cannabinoid antagonists often displayed branched processes extending in all

directions. This is also consistent with the previous study on fixed brain slices taken from animals injected with a single dose of CB1 and CB2 antagonists; this caused a significant decrease in the length of the leading process on migratory neuroblasts in the RMS, together with an increase in the percentage of cells displaying secondary branching (Oudin et al, 2011a). Indeed, a single leading process is crucial for efficient nucleokinesis and so essential for neuroblast migration (Marin et al, 2010). The leading process of a migrating neuron acts as the compass which selects the direction of migration in response to chemotactic cues. A migrating neuron with branched processes often means the cell is in an 'exploratory state' and hasn't decided which direction to follow (Cooper, 2013; Marin et al, 2010). Treatment with CB1/2 receptor antagonists caused the cells to be more branched which means the cells stayed longer in an 'exploratory state'. Moreover, the cells with the leading process oriented other than towards the OB are more likely to migrate to other directions. As a result, the overall migration of the cells towards the OB was significantly reduced with CB1/2 receptor antagonist treatment. In summary, these results clearly support the hypothesis that the CB1/2 receptors regulate neuroblast migration in the intact RMS.

The above results provide clear evidence of an important role for the eCB system in the regulation of neuroblast migration within the RMS. However, we know the neuroblast migration along the RMS is one of the longest migration processes in the mammalian brain, connecting the SVZ of the Lateral Ventricle and OB. However, it should be noted that key factors like developmental stages, architecture environments or neuronal compositions are all fundamentally different between SVZ and OB (Doetsch & Alvarez-Buylla, 1996; Ming & Song, 2005; Ming & Song, 2011) and it is possible that this could be the basis for establishing long-range gradients of chemoattractants or chemorepellants along the RMS. With this in mind we have tested if the eCB system regulates neuroblast migration in a similar manner at both the beginning (close to the SVZ) and end (close to the OB) of the stream and we monitored the directionality of neuroblast migration by calculating the "meandering" index. In the RMS close to the SVZ, treatment with CB receptor antagonists resulted in the increase of the number of exploratory neuroblasts and decrease in the number of cells migrating in a directed manner, with very similar results seen with neuroblasts sampled at the end of the stream. The migratory neuroblasts followed a random rather than directed pathway when eCB signalling was inhibited, and this effect was seen in both beginning and end of the RMS. Thus, we can conclude that the eCB signalling is required for neuroblast motility and guidance all along the RMS.

Having shown that the CB receptors are important for the neuroblast migration, next we wanted to test if other components of the eCB system also play a role in the RMS. Our previous studies show co-expression of the DAGLs and of the CB1 receptor in the same SVZ-derived neuroblasts (Oudin et al, 2011a) suggesting that 2-AG might drive the migratory response. A DAGL inhibitor, OMDM-188 (Ortar et al, 2008) was therefore used in cultured brain slices to block the activity of DAGL in the RMS to test this. The DAGL inhibitor had similar effects on migration as the CB1/2 antagonists. When the DAGLs were inhibited, the cells spent more time immobile, the net migration towards the OB was significantly reduced and total cell displacement was reduced compared to control cells. We also looked at the effect of inhibiting DAGLs on different regions of the RMS, and again similar results were obtained as compared to the CB receptor antagonists. Treatment with the DAGL inhibitor increased the number of exploratory neuroblasts at the expense of the number of cells migrating in a directed manner, with similar results seen at both ends of the RMS. Thus, the results clearly support the hypothesis that the DAGLs regulate neuroblast migration in the intact RMS. We can conclude that DAGL-dependent eCB signalling is required for neuroblast motility and guidance within the RMS and that this pathway operates all along the RMS.

We initially used drugs to inhibit eCB signalling to test the importance of the pathway for neuroblast migration. There are also highly selective agonists available for the CB1/2 receptors and it was of interest to determine what they might do within the relatively intact RMS. A combination of CB1/2 agonists (ACEA and JWH-133) in cultured brain slices was used to test this. The results showed that the CB agonists did not perturb guidance within the RMS, with the only obvious effect on migration being a slight increase in speed. Thus, a long-range gradient of eCB along the RMS is unlikely, as treatment with exogenous CB agonists would ‘flatten’ any potential gradient and perturb guidance. Previous work has shown co-expression of the DAGLs and CB1/2 receptors in the same SVZ-derived neuroblasts (Oudin et al, 2011a; Sutterlin et al, 2013), suggesting 2-AG might act in an autocrine or paracrine way for neuroblast migration. Another study has shown that cell-autonomous 2-AG signalling regulates neurite outgrowth (Keimpema et al, 2013a). Since the neuroblasts migrate over each other in chains, it is possible that 2-AG might exert paracrine effects on neighbouring cells. However, it has been shown that 2-AG has a short half-life and does not diffuse over long distances (Rouzer et al, 2002), therefore a long-range 2-AG gradient in the RMS is unlikely. Thus we postulate that 2-AG does not directly impart

positional information by forming a short or long-range gradient. A more recent study has shown that the eCB signalling can modulate the activity of actin-binding proteins, like fascin, which is important for the polarized morphology of the RMS neuroblasts (Sonego et al, 2013a). We therefore postulate that the eCB signalling is important for maintaining the polarized morphology of the neuroblast by acting on the cytoskeleton proteins. It stabilises the leading process (Oudin et al, 2011a) to allow the neuroblast to read and/or respond to bona fide guidance cues. However, the key observation from the CB receptor agonist treatment is that the agonists do not have significant effect on guidance within the stream, probably because the eCB signalling is already saturated in the RMS. These results provide substantive evidence against the notion that long range eCB gradients might operate along the RMS.

In the present study we used live imaging to determine if and how eCB signalling regulates migration in the RMS within intact brain slices. As well as providing novel insights into how the eCB system regulates migration, the present study highlights some unresolved issues. One of the most pertinent questions is that what actually drives eCB tone in the migratory neuroblast and what is their relative effect in the RMS compared to the eCB system? There is ample evidence for direct cross-talk between FGFR, TrkB and eCB signalling (Berghuis et al, 2005; Lemtiri-Chlieh & Levine, 2010; Maison et al, 2009; Williams et al, 2003). Previous studies have indicated that FGF, BDNF and their receptors –FGFRs and TrkB are expressed in the SVZ-OB system, also FGF and BDNF have motogenic effect on neuroblasts in RMS explant cultures (Chiaramello et al, 2007; Garcia-Gonzalez et al, 2010). However, there has been no direct comparison between the roles of eCB and FGFR or TrkB signalling in the intact RMS and this was therefore picked on as the topics for the chapter 4 and 5 of this thesis and detailed comparison will be discussed below.

In summary, by using time-lapse imaging to characterise the role of eCB system in the regulation of neuroblast migration, this part of the study provides detailed insights into the consequences of inhibiting or activating eCB function on neuroblast migration in the intact RMS. The DAGL-dependent eCB signalling regulates neuroblast migration as well as guidance in the intact RMS, and this effect operates along the entire RMS. Thus we conclude the eCB system is an important regulator of RMS neuroblast migration in the postnatal mouse brain.

7.3 FGFR signalling on neuroblast migration in the RMS

The role of FGF-2 and FGFRs on cell proliferation and neurogenesis in the SVZ has been well studied (Frinchi et al, 2008; Mudo et al, 2007). For example, it has been reported that FGF-2 is expressed in GFAP positive cells and may act on the FGFR1 which was found to be expressed in nestin positive neural stem cells in the SVZ (Belluardo et al, 2008; Mudo et al, 2007). By using in situ hybridization and double labelling together with BrdU administration, a study reported that both FGFR1 and FGFR2 mRNAs are expressed in proliferating precursor cells in the SVZ and these FGFRs may respond to FGF-2 released by non-proliferating cells in the SVZ (Frinchi et al, 2008). The expression of FGFRs and the localisation of FGF-2 in the SVZ-RMS-OB migration route have also been reported on in detail in another study (Garcia-Gonzalez et al, 2010). Immunostaining of the dissociated neural precursors revealed that SVZ derived neuroblasts express the FGFR1. Treatment of explants with FGF-2 significantly increased the migration of neuroblasts from SVZ derived explant cultures, and this effect was inhibited by a selective FGFR1 blocker, SU5402, suggesting an important role of FGF-2 and FGFR1 on neuroblast migration (Garcia-Gonzalez et al, 2010). Moreover, stronger labelling of FGF-2 in the SVZ than in the RMS and OB has been reported by using a FGF-2 antibody, leading the authors to suggest that a caudal-rostral gradient of FGF-2 exists in the neuroblast migration route in the RMS (Garcia-Gonzalez et al, 2010). Thus high levels of FGF-2 emanating from the SVZ might form a gradient to drive neuroblast migration in the RMS. However, the same study also showed that the immunostaining for FGF-2 decreased at P15 with only the SVZ showed a strong expression, suggesting that if this FGF-2 gradient does exist, it is transitory (Garcia-Gonzalez et al, 2010).

In this study, we first treated the brain slices with two specific FGFR inhibitors to block FGFR activity in the RMS. These small molecules bind directly to the FGFR and block activity by competing for ATP binding (Gavine et al, 2012; Mohammadi et al, 1998; Tucker et al, 2014); they will block receptor function irrespective of the nature of the ligand. Our initial study on the descending arm of the RMS revealed results that very similar to those seen with eCB receptor antagonists. Cells stayed a longer time in an immobile state and had shorter displacement and the number of cells migrating towards the OB was also significantly reduced by both. However, when we looked at the different regions of the RMS, remarkably different effects can be seen between the regions. Inhibiting FGFR signalling disrupted cell migration towards the beginning of the RMS, more cells migrated in an exploratory rather directed manner. However, this effect was

not seen at the end of the RMS with the percentage of cells in the “exploratory” and “directed” group relatively unchanged comparing to the control. Therefore, results from inhibiting FGFR signalling support the role of FGFR system on neuroblast migration, but also quickly highlight differences with eCB receptors as the FGFR pathway appears to only operate at the beginning part of the RMS.

The above results have shown the importance of the FGFR activity on neuroblast migration. To test which ligands are likely to be responsible for the effects we have seen above, we used a FGFR1-Fc in brain slice cultures as this small molecule can trap FGF ligands that bind to the FGFR1. FGFR1-Fc is a soluble recombinant protein that contains the extracellular domains of FGFR1 fused to the Fc region of human IgG1. A similar FGF ligand trap has been reported to be used in clinical trial to inhibit tumor growth and angiogenesis (Turner & Grose, 2010). In this study, use of FGFR1-Fc resulted in very similar effects as seen with the FGFR inhibitors. Disruption of cell migration was observed at the beginning of the RMS, while no significant effect can be seen at the end of the RMS. This results support the view that FGFR1 ligands are important for the RMS neuroblast migration.

Next we wanted to test which FGFR1 ligand is important in the RMS. As discussed previously, FGF-2 is the most abundant FGF in the CNS and is able to promote neuroblast migration out of the RMS explant (Garcia-Gonzalez et al, 2010; Zechel et al, 2010), we therefore used a specific FGF-2 neutralizing antibody to block FGF-2 in brain slice cultures. bFM-1 is a very selective monoclonal antibody that blocks FGF-2 signalling and does not cross-react with FGF-1 (Matsuzaki et al, 1989). After incubation of bFM-1 with the brain slice cultures, a large percentage of cells exhibited random migration and change their direction of migration frequently. Again, detailed analysis showed that disruption of the migration was only statistically significant at the beginning of the RMS. Thus, these results support the role of FGF-2 on neuroblast migration in the RMS and FGF-2 is likely to be the ligand responsible for the FGFR signalling in the RMS.

A previous study using an anti-FGF-2 antibody for immunostaining on brain slices reported that FGF-2 is expressed at higher level in the SVZ than in the RMS and OB resulting in a ligand gradient along the RMS (Garcia-Gonzalez et al, 2010). The results of our study are clearly in keeping with this hypothesis. However, we have not been able to reproduce this result using some available anti-FGF-2 antibodies, possibly due to sensitivity issues and/or the loss of FGF-2 proteins from the slices during immunostaining and/or as a consequence of fixation. Therefore we

tested the hypothesis indirectly by adding high concentration of exogenous FGF-2 into brain slice cultures as this would be expected to saturate and disrupt any endogenous FGF-2 gradient that exists in the RMS. Results here showed that adding exogenous FGF-2 to the brain slice cultures disrupted the migration at the beginning of the RMS and cells migrated in a more random as opposed to directed manner. These results together with the FGF-2 neutralizing antibody results suggested that a certain concentration of FGF-2 at the beginning of the RMS was required for directed neuroblast migration, exogenously added FGF-2 might destroy a gradient of FGFs within the RMS with the result being that cells lose positional information; they can still move, but lose their sense of direction. This is consistent with the hypothesis that there might be a caudal-rostral FGF-2 gradient along the SVZ and RMS that drives the migration of the neuroblasts by working on FGFRs and downstream pathways. Indeed, gradient of FGFs, especially FGF-2, has been reported to be involved in many physiological processes including embryogenesis, limb bud, neural development, neuronal differentiation and neurogenesis (Bokel & Brand, 2013; Dyer et al, 2014; Keenan et al, 2012; Lahti et al, 2011; Wu et al, 2014a). Therefore our results support the existence of a FGF-2 gradient in the RMS and this gradient is likely to drive the neuroblast to leave the SVZ and migrate in the RMS. Nonetheless, an important caveat of the exogenous FGF-2 administration experiment is that responses to this growth factor are often biphasic with higher concentrations acting like an inhibitor possibly due to receptor and/or pathway desensitisation (Williams et al, 1994b; Williams et al, 1995). Thus in our experiment the excess FGF-2 added into the slice cultures might have inhibited the receptor, rather than flattened a ligand gradient.

From our results of time-lapse movies, all the treatments targeting FGFRs or FGF-2 have a significant effect on migration behaviour at the beginning part of RMS. The cells become more exploratory, change their directions frequently which resulted in a reduction in the total displacement. However, the actual distance the cells migrated was not significantly changed. There was a small but significant effect on the speed of migration by inhibiting FGF-2 with the neutralizing antibody. However, the average speed of neuroblast migration was not significantly altered by FGFR inhibitors or FGFR1-Fc. Thus we postulate that the FGFR signalling in the RMS (at least at the beginning part of the RMS) is important for the cells to sense the guidance cues in the local environment thus maintain the momentum to migrate toward the OB.

While all the treatments targeting FGFR signalling resulted in the neuroblasts migrating more randomly at the beginning of the RMS, no significant effect was seen towards the end of the RMS.

This is consistent with the hypothesis that a FGF-2 gradient might exist in the RMS. The observation at the end of RMS also serve as an internal control; these indicate that the effect of the treatments like FGF-2 antibody and FGFR1-Fc chimera at the beginning part of RMS is not caused by toxic or non-specific effects on neuroblasts.

The above results provide a very substantial body of evidence for FGF-2/FGFR function regulating RMS migration in a slice culture. Similar support for the importance of eCB signalling was obtained from a study treating animals with a range of CB1/2 antagonists for 24 h and showing that this resulted in changes in neuroblast morphology within the RMS, most notably a decrease in the length of the leading process and an increase in secondary branching (Oudin et al, 2011a). Here we reported a similar 24 h treatment with the FGFR inhibitor AZD4547 also resulted in morphological changes on the neuroblast in the RMS. Again, the leading process length was significantly shorter after AZD4547 treatment as compared with the control treated animals. A shorter process length might compromise the neuroblasts ability to sense the local environment for guidance cues, and therefore the direction of migration (rather than migration per se) might be compromised. Remarkably, when we check the process length in detail by dividing the RMS into 4 regions, a gradated effect was seen with cells towards the beginning of the stream showing a highly significant decrease in leading process length, and cells at the distal end of the stream showing no response. Thus our results provide evidence for FGFR signalling regulating neuroblast morphology *in vivo*, and support the conclusion made with slice cultures pointing to regional differences in FGFR signalling within the RMS.

The above results all support the hypothesis that a FGF-2 gradient might exist in the RMS to regulate neuroblast migration, however in this study the direct antibody staining method was unable to demonstrate such a gradient. To further look for the evidence for the existence of FGF-2 gradient, we turned to the RT-qPCR method to quantify the transcript expression levels of FGF-2 and its receptor FGFR1 in different regions of RMS. Our RT-qRCR results showed that the FGF-2 transcripts were expressed at higher level in the SVZ tissue compared to the OB, while the FGFR1 transcripts level showed no significant difference. This is consistent with the previous study where they showed higher levels of FGF-2 in the SVZ than the OB (Garcia-Gonzalez et al, 2010). However, transcripts encoding FGFR1 were similar at both regions. Previous studies have reported that the embryonic development of OB is independent of SVZ and RMS, even cortex, and FGFR1 is required for OB morphogenesis (Garcia-Gonzalez et al, 2010; Hebert et al, 2003;

Nishizumi & Sakano, 2015). Conditional knock out of FGFR1 at early stage in the telencephalon of mice resulted in the absence of OB, but the cells can still migrate from SVZ to an OB like protrusion (Hebert et al, 2003). Therefore, we postulate that the FGFR1 may have different functions close to the SVZ and in the OB. While the FGFR1 signalling regulates neuroblast migration close to the SVZ, it might act as a signal important for morphogenesis in the OB but unrelated to the cell migration. Thus we can conclude that the transcripts level of FGF-2 showed here provide basis to support the existence of putative caudal-rostral FGF-2 gradient in the RMS, however more accurate methods for determining the receptor and ligand levels will be needed to further support this evidence, especially as the difference that we found is relatively small.

To substantiate the above results, we also studied migration in a model where in principal we conditionally knock-out both the FGFR1 and FGFR2 from migratory neuroblasts. Conditional Fgfr1/2 $^{flox/flox}$ mice were bred by crossing Fgfr1 and Fgfr2 conditional knock out mice (Xu et al, 2002; Yu et al, 2003; Yu et al, 2011). Electroporating a pCAG-Cre-IRES-EGFP plasmid into the lateral ventricle of these mice in principle should excise the sequences flanked by LoxP sequences employing the Cre-LoxP system. However, conditional knock out of FGFR1/2 by electroporating a Cre-containing plasmid into the lateral ventricle did not result in significant morphological change in the RMS 5 or 10 days later. The possibility that the knock out strategy might have failed needs to be considered. The results showed the expression of the GFP protein in the neuroblasts along the RMS either 5 or 10 days after electroporation, this should mean the Cre recombinase was introduced into the same population of cells. However, since these mice were maintained on a mixed genetic background and there were multiple LoxP sequences (at least 4 sites were flanked by LoxP sequence on both sides) existing in the genome, there was chance that there might not be enough copies of Cre recombinase available in the neuroblasts to completely knock out both Fgfr1 and Fgfr2 alleles. Also a previous study has reported that even if appropriate DNA excision takes place in two β1 intergrin transgenic LoxP mice lines, different outcomes have been seen with one model showed no change in β1 intergrin protein level 28 days later (Turlo et al, 2010). Therefore it is possible that even if the receptor genes have been deleted, FGFR1 and FGFR2 proteins may persist and still function in these cells at the time we checked the cell morphology. Unfortunately antibody staining in slice cultures is unlikely to have the sensitivity and resolution to be useful in determining if the strategy has worked or not. If we assume that the knock out strategy did work, and that we have tracked neuroblasts that lack both

FGFR1 and FGFR2, there are other reasons why the results obtained might have differed from those seen with the pharmacological and other reagents that disrupt FGF-2/FGFR signalling. For example, a previous study has showed that the gradient effect of FGFs on cell migration operates in a collective manner on large population of cells rather than at the level of an individual cell (Benazeraf et al, 2010). With “chain” migration in the RMS being driven by cell-cell interactions between the neuroblasts (Garcia-Verdugo et al, 1998; Wang et al, 2011; Wozniak & Bruska, 1999), when adding FGFR inhibitors or FGF-2 or its’ neutralizing antibody into the brain slices, we clearly target FGFR signalling in the whole population of the cells in the RMS. This contrasts with electroporation as this generally only result in sparse labelling (5-30%) in the RMS (Boutin et al, 2008; Sonego et al, 2013b). Therefore knock out by electroporation may not be sufficient to alter the graded FGFR signalling in the RMS as cells that lack the receptors might remain instructed by cells that continue to express the receptors. Another explanation for the divergent results might be the neuroblasts may adapt to the relatively chronic loss of FGFR signalling by compensatory mechanisms. Indeed, previous studies have reported on the ability of neural stem cells to rapidly adapt to the loss of FGFR signalling (Sutterlin et al, 2013). In this context, the Cor-1 neural stem cell line, derived from E16.5 mouse cortex, shares many properties with the NSCs and migratory neuroblast that we study in the slice cultures (Conti et al, 2005; Pollard et al, 2006). For example, the proliferation of this cell line can be inhibited by a DAGL inhibitor or CB1/2 antagonists, the same drugs can also inhibit the proliferation of SVZ neuroblasts (Goncalves et al, 2008). In addition, eCB signalling has been reported to regulate Cor-1 cell migration in a wound assay and the same signalling system also regulates neuroblast migration in the RMS (Oudin et al, 2011a). Thus, this neural stem cell line would appear to be a good model for understanding how the FGFR and other receptor systems cooperate to regulate neurogenesis and cell migration and also for determining how the cells adapt to loss of a pathway. For example, a previous study in our lab reported that inhibiting FGFR signalling in Cor-1 cells using PD173074 resulted in a very rapid reduction (~60%) of phosphorylation of ERK1/2 in these cells seen after 30 min, and importantly this pathway has been implicated in cell proliferation and migration (de Graauw et al, 2006; Turner & Grose, 2010). However, even in the continuous presence of the FGFR inhibitor, phosphorylation of ERK1/2 returned to control level within 3 h (Sutterlin et al, 2013). Thus this study showed that the Cor-1 cells were able to quickly adapt to the loss of FGFR signalling (Sutterlin et al, 2013). Therefore the conditional knock out of FGFR1/2 may have also resulted in

the neuroblasts initiating the rapid adaptation mechanism and compensated for the loss of FGFR signalling *in vivo*.

The results above were all obtained from postnatal mice. As the neuroblast migration in the RMS persists into adulthood, the time point that might need more therapeutic intervention in the brain, it will be interesting to further test the role of FGFR signalling in mature adult mice. However, the study of adult animals is more challenging due to the fact that the GFP signal in the labelled neuroblasts is lost 2-3 weeks after the initial electroporation (Boutin et al, 2008; Sonego et al, 2013b) and the electroporation of older mice is relatively inefficient at labelling neuroblasts in the RMS (Barnabe-Heider et al, 2008). Therefore in this study, I did some preliminary experiments to establish a model that would allow for the future study on the neuroblast migration in the RMS in adult animals. Conditional knockout mice employing the LoxP system caught our attention and in particular a ROSA26-YFP mouse line employing the Cre-LoxP system that had been reported previously to be able to permenantly label a subpopulation of cells (Lacar et al, 2010; Platel et al, 2010). More specifically, the ROSA26-YFP mice genome contains a Stop sequence flanked by LoxP between the ubiquitous Rosa26 promoter and YFP encoding sequence. Electroporation of a Cre-expressing plasmids into the laterventrical of ROSA26-YFP mice will introduce the Cre recombinanse into a population of stem cells in the SVZ. Thus, the Cre-LoxP system will excise the Stop sequence and induce the permanent genomic expression of YFP in these stem cells which in principle will subsequently generate a continuous population of YFP-expressing migratory neuroblasts that will migrate from the SVZ to the OB well into adulthood. Results from our data using ROSA26-YFP mice showed successful labelling of the neuroblasts in the RMS up to 8 weeks following the initial electroporation, however, longer time point labelling is also possible and has been reported elsewhere (Platel et al, 2010). We also did some preliminary expriments on 6 weeks old (general regarded as young adult mice) ROSA26-YFP mice to test the role of FGFR signalling. Our preliminary data showed that a 24 h treatment with FGFR inhibitor AZD4547 resulted in branched cell morphology in the RMS, and there was a tendency of shorter process length after treatment. Thus we established in our lab a model for successful labelling of neuroblasts in young adult mice and by using this model our pilot study supports the role of FGFR signalling on neuroblast migration in the RMS in young adult mice. However, more experiments will be needed to confirm the role of FGFR signalling in adulthood.

In summary, our results suggest that FGF-2/FGFR signalling play a very important role in driving the migration of neuroblasts at the beginning of the RMS, but not at the end part. Our results suggest that FGFs within the RMS provide positional information that is “read” by FGFRs on the neuroblasts to direct them to the bulb. The observation that FGFR function is higher in the SVZ and decreases toward the OB might be explained by the existence of a FGF-2 gradient in the RMS which is important for the neuroblasts to leave the SVZ and migrate in the RMS. A matrix-bound gradient of FGF-2 has been reported to provide positional information in other systems (Wu et al, 2014a). However, we have not been able to confirm the existence of such a gradient. Mechanisms other than a FGF gradient might also explain the above results, for example, there are feedback mechanisms within the cell that can regulate FGFR activity, including “sprouty” proteins (Cabrita & Christofori, 2008; Ching et al, 2014; Mason et al, 2004), and it remains possible that these operate in a graded manner along the RMS.

7.4 TrkB signalling on neuroblast migration in the RMS

The TrkB receptor is a member of a tyrosine kinase receptor family that includes TrkA and TrkC. TrkB is the high affinity receptor for BDNF and NT-4, and binds less efficiently with neurotrophin-3 (NT-3) (Huang & Reichardt, 2001). The TrkA receptor binds NGF while TrkC is activated only by NT-3 (Huang & Reichardt, 2001; Skaper, 2012). Therefore, the TrkB receptor mediates multiple effects of neurotrophic factors, which include promoting neuronal differentiation and survival (Huang & Reichardt, 2001). As mentioned above, the effect of TrkB on neuroblast migration in the RMS has been reported, and this effect is likely to be attributable to BDNF serving as the ligand as it is present throughout the RMS (Chiaramello et al, 2007; Snapyan et al, 2009). The NGF-TrkA pathway also regulates many physiological processes including neuronal growth and survival and NFG-TrkA complex endocytosis has been implicated in the signal transduction of the neurons (Grimes et al, 1996; Skaper, 2012).

Although a previous study has already shown that BDNF has a motogenic effect on neuroblasts in RMS explant cultures (Chiaramello et al, 2007), conflicting studies have appeared on the role of BDNF in the RMS. A study showed that both the speed of migration and the number of neuroblasts migrating towards the OB were increased by using an antibody reported to block BDNF function (Bagley & Belluscio, 2010). However, these results are not obviously compatible with another study that reported BDNF promotes the migration of SVZ neuroblasts, acting both as inducer and attractant through TrkB activation. Also pharmacological blockade of BDNF

dependent pathways on SVZ explants impairs neuronal migration (Chiaramello et al, 2007). In agreement with the conclusion that TrkB signalling is required for migration, it has been reported that neuroblast migration in acute brain slices from adult mice is inhibited by incubation with TrkB-Fc (Snapyan et al, 2009). We have revisited the role of TrkB-Fc on neuroblast motility and guidance along different regions of the stream in the intact brain slices to not only clarify its role, but also allow for a direct comparison of the results seen with agents that block eCB signalling. By doing so we wanted to see if there is any basis for postulating interplay between TrkB and eCB signalling in the regulation of migration in the RMS

We also used TrkB- and TrkA-Fc chimeras on brain slices of intact RMS followed by time-lapse imaging to test the relative importance of the respective neurotrophin receptors on the neuroblast migration in the RMS. Again, *in vivo* postnatal electroporation and subsequent acute brain slice preparation followed by time-lapse imaging was used to visualize and analyze the migration dynamic of the neuroblasts after TrkB-Fc and TrkA-Fc treatment. Our results clearly support an important role for TrkB signalling on neuroblast migration in the RMS. The cells treated with TrkB-Fc were less mobile and the average speed of migration was also decreased when compared to the control cells, supporting the role of TrkB signalling on motility of the neuroblast migration. The percentage of cells migrating towards the OB and the net displacement of the cells were also reduced, supporting the role TrkB on guidance in the RMS. Our results also suggest that TrkB signalling regulates migration throughout the RMS because the treatment with TrkB-Fc increased the percentage of exploratory cells at both beginning and end of the RMS. Our results also show that the effect of the TrkB-Fc on migration guidance at the end of RMS was just as substantial as its effect at the beginning, which is of interest given that a real-time RT-PCR study has shown both BDNF and TrkB receptors expressed at lower level in the SVZ compared with OB extracts (Chiaramello et al, 2007). Such different levels of receptor and / or ligand in the RMS might have been expected to lead to a graded response along the RMS, as have been suggested for slits and GDNF that act as chemorepellents or chemoattractant respectively in the RMS (Hu, 1999; Paratcha et al, 2006; Wu et al, 1999). However, a graded response for TrkB signalling was not observed in the present study as inhibiting the pathway showed similar effects at both ends of the RMS. Thus our results clearly support the notion that TrkB signalling has an important function on neuroblast migration in all regions of the RMS, and this is in agreement with results reported by others that includes a report that TrkB receptors are expressed by migrating neuroblasts in

different regions of the RMS (Bath et al, 2008; Chiaramello et al, 2007). Our data not only confirm a motogenic role for TrkB (Chiaramello et al, 2007; Snapyan et al, 2009), but also extend its function as a local guidance cue throughout the stream.

Our results clearly support the role of TrkB signalling in the RMS, but it did not directly address the identity of the ligand. As TrkB receptor can bind to both BDNF and NT-4 in high affinity, the use of a TrkB-Fc can potentially inhibit both neurotrophins. Indeed, by both working through TrkB receptor, BDNF and NT-4 can exert very similar effects as well as differentiated effects in the CNS (Bosco & Linden, 1999; Heppenstall & Lewin, 2001; Hyman et al, 1994; Polleux et al, 2002). For example, a study using embryonic mice medial ganglionic eminence (MGE) explant co-cultured with postnatal rat cortical slices showed that BDNF and NT4 have similar effects on tangential migration of the interneurons from the MGE (Polleux et al, 2002). However, another study on retina ganglion cells (RGCs) indicated that BDNF and NT-4 had very different effects on modulating neurite outgrowth, BDNF supported mainly polarized outgrowth, whereas NT-4 induced the appearance of intensely branched symmetrical arbours (Bosco & Linden, 1999). An electrophysiology and knock out mice study also showed that although NT-4 was normally present in the mouse spinal cord, it was BDNF but not NT-4 that was required for normal flexion reflex plasticity and function (Heppenstall & Lewin, 2001). In this study, BDNF is more likely to be the key ligand for the TrkB receptor in the RMS based on the previous studies showing that BDNF was able to promote dendritic development of SVZ-derived neuronal precursors and was essential for their survival in the OB (Gascon et al, 2005; Kirschenbaum & Goldman, 1995; Linnarsson et al, 2000). Intraventricular administration of BDNF resulted in a substantial increase in the number of newly generated cells within the migratory pathway and in the OB (Pencea et al, 2001). Moreover, both BDNF mRNA and protein were expressed throughout the RMS in young adult mice, and BDNF was able to promote neuroblast migration out of the explant of RMS from P4-10 mice and this effect was inhibited by a TrkB inhibitor, K252a (Chiaramello et al, 2007). Therefore in this study where P7-P9 mice were used for brain slices which was consistent with the time point (P4-P10) in the previous study (Chiaramello et al, 2007), it is more likely to be BDNF rather than NT-4 that regulates neuroblast migration in the RMS. However, the role of NT-4 in the RMS cannot be ruled out and future study will be required to determine if it is expressed in the RMS and if yes, whether it regulates migration.

While treatment with TrkB-Fc disrupted migration in the RMS, our results showed that treatment with TrkA-Fc did not have a significant effect on the neuroblast migration in the RMS, suggesting that this receptor has little if any function in the migratory neuroblasts in the RMS. Indeed, the TrkA-Fc treatment in this study didn't result in any significant effect on any of the parameters measured on the migration including the average speed. However, the results with the TrkA-Fc can be taken as a good internal control for the TrkB-Fc, as they demonstrated that Fc-chimeras do not have non-specific effects on any of the measured parameters. However, it is perhaps worth noting that there was a clear trend towards an effect for TrkA-Fc at the beginning of the RMS, leaving open the possibility that NGF and the Trk-A receptor might play a small role in migration. Further studies examining the expression of this ligand and receptor pairing would be required before developing this hypothesis further. Likewise, studies on the effects of NGF on neuroblast migration from RMS explant cultures would provide an alternative methodology for exploring the hypothesis. The relative lack of effect of the TrkA-Fc compared to the TrkB-Fc argues against a non-specific effect of the Fc chimeras on migration. Others have used the same tools and demonstrated differential results. For example, it has been shown that it was TrkB-Fc, not TrkA-Fc or TrkC-Fc, contributed to the development of kindling (Binder et al, 1999). Nonetheless, it would be prudent to use additional tools in future studies to further test the importance of TrkB signalling for neuroblast migration. This was not deemed to be a priority in this study as a previous study using a TrkB-Fc as well as TrkB conditional knock-out mice has shown the importance of TrkB signalling on neuroblast migration in adult mice (Snapyan et al, 2009).

In summary, by using live-cell imaging on the intact brain slices, the results suggest that the TrkB signalling is an important regulator of RMS neuroblast migration, while NGF-TrkA does not seem to play a role on the neuroblast migration in the RMS. Acute disruption of TrkB signalling disrupted neuroblast motility and guidance at both end of the RMS, also TrkB signalling had a small but significant effect on the speed of neuroblast migration in the RMS.

7.5 Comparison between eCB, FGFR and TrkB signalling on neuroblast migration

Our results show that the eCB system can regulate both neuroblast motility and local guidance throughout the stream. By comparing these results to the FGFR system, we can see similarities as well as very clear differences between these two signalling systems in regulating the

neuroblast migration. One common effect between targeting the FGFR and eCB signalling is that the effect on migration is manifest as a loss of directional movement towards the OB, as opposed to a loss of migration per se. Treatments inhibiting the eCB system or targeting the FGFR signalling in brain slice cultures resulted in the cells to migrate in a more exploratory rather than directed manner. The cells seem to have lost their direction of migration and change their direction frequently, as a result, the percentage of cell migrating towards the OB and cell displacements were all reduced. However, when the cells do migrate the speed of migration was not significantly different from controls in most treatments. As discussed above, pharmacological inhibition of the eCB and FGFR signalling *in vivo* all resulted in the cells exhibiting shorter process lengths. Thus we postulate that both eCB and FGFR signalling might be important for maintaining the polarized morphology of the neuroblast in the RMS by stabilising the leading process to allow the neuroblast to read and/or respond to guidance cues. Apart from the similarities, the differences between these two signalling systems are very clear. The main difference is the regional effects of the FGFR signalling. Targeting FGFR signalling only affected neuroblast migration at the beginning of the RMS, while inhibiting the eCB system did not show regional differences. Also we showed that the eCB agonists did not significantly affect the directed cell migration in the RMS. Unlike the eCB system, results in the FGFR chapter showed that either inhibiting FGF-2 by a specific neutralizing antibody or adding exogenous FGF-2 to the brain slice cultures disrupted the migration at the beginning of the RMS and cells migrated in a more random as opposed to directed manner. Also the pharmacological inhibition of FGFR signalling *in vivo* showed regional specific effects on neuroblast morphology, while inhibiting the eCB signalling *in vivo* affects the neuroblast morphology throughout the RMS (Oudin et al, 2011a). Based on the differences above, it's very unlikely that these two pathways directly interact to regulate migration in the RMS, especially at the end of the RMS where the FGFR signalling is not important for directed cell migration.

Targeting TrkB signalling resulted in defects in neuroblast migration that are similar to those seen following targeting of eCB signalling. Both treatments disrupted both motility and guidance, resulted in the cells to spend more time immobile, and migrating in an exploratory rather than directed manner. The only difference seemed to be the effects on the average speed of migration since the TrkB-Fc incubation slightly decreased the speed while the eCB inhibitors did not show significant effect, suggesting the TrkB signalling is more important for the motility. Based on the

above results that eCB and TrkB signalling perturbation had similar effects on neuroblast migration in the RMS, and evidence from the previous studies that eCB and TrkB signalling interact with each other in many different ways in other neuronal types (see above for details), it will be very interesting to investigate the direct linkage of the two systems on the intact brain slices. However, it might be very difficult to interpret the results from the combined treatment of eCB and TrkB inhibitors on brain slices, as inhibiting eCB or TrkB signalling on their own already resulted in substantial or maximal disruption on migration and further disruption is unlikely. For example, the percentage of cells migrating towards the OB decreased from ~70% to ~40% in both eCB antagonists and TrkB-Fc treatment and these effects were already maximal because further reduction in the cells towards the OB again means directed migration, but towards the opposite direction rather than to the OB. Using *in vitro* explant cultures from the RMS might be a better approach to test the combined effect of eCB and TrkB since the migration rate out of the explant can be easily measured. In this context, preliminary results showed that the maximal distance of the neuroblast migration from the explants can be inhibited by CB1/CB2 antagonists (Oudin et al, 2011a) but not by a TrkB inhibitor K252a (Doherty lab, unpublished observation), suggesting that eCB and TrkB signalling operate independently in neuroblast migration. Nonetheless, given that there is extensive cross-talk between these two pathways and that inhibiting them independently results in very similar perturbations in migration within the RMS, it would appear likely that future studies might reveal some level of interaction between them.

In summary, our results provide detailed insights into the consequences of inhibiting eCB, BDNF and FGFR function on neuroblast migration in the intact RMS. The DAGL-dependent eCB signalling is important for RMS neuroblast migration. The eCB and TrkB signalling regulate both neuroblast motility and local guidance throughout the stream. Acute disruption of TrkB signalling had similar effects as inhibiting DAGL-dependent eCB signalling on motility and guidance at both end of the RMS, with the only difference being that TrkB signalling had a small but significant effect on the speed of migration that was not seen when eCB signalling was blocked. The FGF-2/FGFR signalling differs from the role of eCB and TrkB signalling by playing a very important role on driving the migration of neuroblasts at the beginning of the RMS, but not at the end part. Thus, in this context these results do not support direct cross-talk between the eCB and FGFR signalling systems on the regulation of neuroblast migration in the RMS, while further study will be needed to test the direct cross talk between the eCB and TrkB signalling. Although not pertinent

to the “cross-talk” question, it is perhaps worth noting that by comparing the effects of these three systems at the beginning of the RMS, we did not see any particular “rank” of these three systems in regulating neuroblast migration as all of them significantly disrupt the cell migration to a similar level.

7.6 Why study DAGL intracellular trafficking?

Having established the eCB signalling is important for migration, and having compared it with two other receptor systems, we next wanted to start to make some inroads into the mechanisms that might regulate DAGL function. There is no shortage of questions that might be addressed, e.g. the nature of the upstream ligand/receptor pairing that drives DAGL-dependent eCB signalling, and the molecular basis for enzyme activation. In the later context, this laboratory has postulated that DAGL activity will be governed by phosphorylation of a regulatory loop that limits substrate access to the active site within the catalytic domain (Reisenberg et al, 2012), and others are studying this question. However, given that location within the cell, and in particular whether the DAGLs are in the same membrane compartment as the upstream receptors and downstream effectors (the CB1/2 receptors), we decided to determine if there is dynamic cycling of the enzymes between an intracellular pool and a pool expressed at the cell surface. At the outset we viewed this as a possible mechanism for regulating the cell surface expression of DAGLα at the post-synaptic spine akin to the recycling mechanisms that govern the availability of neurotransmitter receptors in the same membrane (Arancibia-Carcamo et al, 2006; Collingridge et al, 2004; Kittler et al, 2005). This is important for synaptic plasticity and in brief is governed by the regulated endocytosis of the neurotransmitter receptors followed by the recycling of the endocytic vesicles back to the cell surface.

Endocytosis is a very general cellular process used to internalize membrane proteins, lipids, extracellular ligands and soluble molecules (Arancibia-Carcamo et al, 2006). It is a basic cellular process that controls the protein and lipid compositions of the plasma membrane therefore regulating how the cells interact with and respond to their environment (Doherty & McMahon, 2009). A variety of endocytic pathways have been discovered and studied so far, however, these pathways are generally divided into two groups: clathrin-mediated endocytosis or clathrin-independent endocytosis. Clathrin-mediated endocytosis is by far the most extensively studied and best understood pathway. Clathrin is a protein that forms a triskelion shape by its heavy and light chain complex; this triskelion can assemble into apolygonal lattice that helps to deform the

plasma membrane into a clathrin-coated pit and subsequently into a clathrin coated vesicle. AP-2 is required for the recruitment of clathrin to the plasma membrane and it provides a link between clathrin and the molecules that will be internalised via the clathrin coated pit (Farsad et al, 2003; Heilker et al, 1996; Traub, 2003; Traub, 2009). Dynamin is also essential for clathrin-coated vesicle formation as it is critical for the pinching and release of a completed vesicle from the plasma membrane (Doherty & McMahon, 2009). Once the clathrin built small vesicles detach from the plasma membrane, they are then transported within cells.

Clathrin-mediated endocytosis is a ubiquitous process which is essential for all eukaryotic cells, it mediates the internalization of extracellular hormones or signalling factors as well as being involved in nutrition uptake and general signal transduction (Arancibia-Carcamo et al, 2006; Miaczynska & Stenmark, 2008). A recent study reported that ~95% of the earliest detectable endocytic vesicles arise from clathrin-coated pits (Bitsikas et al, 2014). Therefore clathrin-mediated endocytosis is regarded as a “housekeeping” pathway by some researchers (Bitsikas et al, 2014; Bradford et al, 2015). Although the majority of molecules are internalized via clathrin-mediated endocytosis, clathrin independent pathways have also been reported (Doherty & McMahon, 2009; Mayor et al, 2014; Miaczynska & Stenmark, 2008). Caveolin and lipid raft-dependent pathways are relatively well characterized clathrin-independent pathways (Arancibia-Carcamo et al, 2006). Pathways that involve Flotillin, GPI-linked proteins, Arf6 and IL2Rβ have also been reported (Doherty & McMahon, 2009; Glebov et al, 2006; Lamaze et al, 2001; Naslavsky et al, 2004). More recent studies reported Endophilin, a protein that has been assigned as a component of clathrin-mediated endocytosis, controls a fast-acting tubulovescicular endocytosis pathway that is independent of clathrin and AP-2 (Boucrot et al, 2015; Renard et al, 2015). However, some clathrin-independent pathways are less well understood, and some proposed pathways remain controversial; some of these might only be functional in special situations like cell migration or in specific cell types like hippocampal neurons (Bitsikas et al, 2014; Doherty & McMahon, 2009; Howes et al, 2010; Mayor et al, 2014; Sandvig et al, 2011). For all the endocytosis pathways, once the molecules or cargoes have been internalized, they enter the endocytic pathway which consists of distinct membrane compartments like endosomes or lysosomes (Arancibia-Carcamo et al, 2006). The early endosome is usually the first sorting station for endocytic pathways; from here the molecules or cargoes will then generally be sorted for degradation or recycling (Jovic et al, 2010).

The DAGLs are key enzymes in the eCB signalling system which is a therapeutic target for many physiological disorders and diseases like epilepsy, pain and neurodegeneration (Ben Amar, 2006; Bisogno et al, 2003; Gao et al, 2010). Within neurons the DAGLs are localized to axons during development, but DAGLα becomes restricted to dendritic spines in the adult brain (Bisogno et al, 2003; Yoshida et al, 2006). The underlying mechanism regulating this polarised compartmentalisation of DAGLα is not known, but is most probably determined by its ability to bind to homer proteins and/or CamKII as these molecules are also assembled into protein complexes that specifically localise to dendritic spines (Jung et al, 2007; Shonesy et al, 2013). Knock out studies have revealed that DAGLα is responsible for DSI/DSE to regulate synaptic plasticity in the CNS (Gao et al, 2010; Tanimura et al, 2010).

The restricted localisation of DAGLα to the dendritic spine underpins the function of eCB signalling in DSI and DSE throughout the nervous system (Bisogno et al, 2003; Gao et al, 2010; Yoshida et al, 2006). Once localised to a post-synaptic spine, vesicular recycling of many other receptors like AMPA, NMDA and GABA(A) receptors can modulate synaptic strength (Arancibia-Carcamo et al, 2006; Kittler et al, 2005). For example, GABA(A) receptors are the major sites of fast synaptic inhibition in the CNS, they have been shown to be constitutively internalized through clathrin-mediated endocytosis in hippocampal neurons (Kittler et al, 2000). Inhibition of GABA(A) receptor internalization can enhance the amplitude of miniature inhibitory synaptic current and whole cell GABA(A) receptor current, indicating the number of GABA(A) receptors expressed on the cell surface of the neurons regulate the efficacy of synaptic inhibition (Kittler et al, 2005; Kittler et al, 2000). As DAGLα also plays an important role in synaptic plasticity, we decided to develop and test the hypothesis that this key enzyme might also be dynamically regulated in spines, perhaps “shuttling” between an intracellular and cell surface location. To this end, an extracellular HA tagged DAGLα construct was generated to enable us to identify the enzyme at the surface of living cells and to follow internalisation and recycling back to the cell surface using classical antibody feeding experiments. Our results show that there are indeed distinct surface and intracellular pools of DAGLα in dendritic spines, and that DAGLα can indeed be internalized in several cell lines as well as in the dendrites of cultured hippocampal neurons.

7.7 DAGLα intracellular trafficking in COS-7 cells

Our results show that DAGLα undergoes constitutive internalization followed by recycling back to the plasma membrane in COS-7 cells. As endocytosis is an energy using process, it is

reasonable to postulate that there is a reason behind this dynamic cycling. It is possible that the internalization of DAGLα is used by cells to control the level of the enzyme at the cell surface and thereby regulate eCB signalling. Indeed, a change of localization of DAGL activity from an intracellular pool to plasma membrane, measured as the release of arachidonic acid (AA) from diacylglycerol (DAG) in human neutrophils, has been reported (Balsinde et al, 1991). In this study the authors simply assayed for a DAGL enzymatic activity and did not characterise a molecular entity. Nonetheless, this change of DAGL activity localization towards the plasma membrane in neutrophils was stimulated by the calcium ionophore A23187 and the translocation of DAGL may play an important role in regulating eCB signalling in stimulated cells (Balsinde et al, 1991). Further study will be required to test the role of DAGLα endocytosis in enzyme activity such as 2-AG production.

It was perhaps not surprising to find that internalized DAGLα co-localized with the early endosome markers EEA 1 and Rab5 as the early endosomes are the first sorting station for several endocytic pathways. (Arancibia-Carcamo et al, 2006; Jovic et al, 2010). As DAGLα can be recycled back to the plasma membrane, it's likely to be sorted to recycling endosomes after the early endosome stage. The failure to detect co-localization of DAGLα with LAMP1 supported this by suggesting that DAGLα was not trafficked to the lysosomes. However, we have not been able to determine which endocytosis pathway is responsible for DAGLα internalisation. Our results failed to show DAGLα co-localization with transferrin, a protein that has been well studied and established to be involved in clathrin-mediated endocytosis, suggesting DAGLα might be internalized by a clathrin-independent pathway. In preliminary experiments with Dynasore, a selective dynamin inhibitor (Macia et al, 2006), or a dominant-negative dynamin-2 (K44A) mutant (Ringerike et al, 1998), we have not seen any obvious alteration in DAGLα internalization (data not shown). These data together suggest that endocytosis of DAGLα might be independent of clathrin and dynamin. However, further experiments will be needed to confirm this conclusion.

Interestingly, a consensus AP-2 binding motif (RRRWS) can be found in the N-terminus of DAGLα, and this might suggest that AP-2 can play a role in DAGLα endocytosis (Gareth Williams, unpublished data). This putative AP-2 binding motif (RRRWS) is conserved across species in DAGLα but is absent from DAGLβ. A similar motif has been reported to be conserved in all GABA(A) receptor beta subunit isoforms and phosphorylation of serine in this motif inhibits the receptor binding to AP-2 therefore inhibiting the GABA(A) receptor internalization (Kittler et al,

2005). We designed a peptide named “peptide 1” corresponding to the first 17 aa of the N-terminal of DAGLα which contains the motif in tandem with an Antennapedia peptide sequence which enables the peptide to penetrate cellular membranes (Bechara & Sagan, 2013; Derossi et al, 1994; Thoren et al, 2000). The Doherty lab has used this strategy to design numerous peptides that can enter cells and compete for protein-protein interactions (Hall et al, 1996b; Saffell et al, 1997; Vastrik et al, 1999). In principal, the cell permeant peptide mimetic of the DAGLα N-terminus should be able to compete out the binding of any protein that might interact with this region of the enzyme, and this might include AP-2. Interestingly, we did see a trend of reduced DAGLα internalization when peptide 1 was added into the cell medium, however the results failed to reach statistical significance. These results suggest DAGLα internalization may be independent of AP-2 or other motifs/regions may be required for DAGLα endocytosis and future studies will be required to confirm this.

7.8 Effects of calcium on DAGLα internalization

Calcium has been implicated both upstream and downstream from the cannabinoid receptors in many studies, for example, DAGL-dependent eCB signalling can couple to CAM / FGFR signalling to regulate axonal growth and guidance by driving calcium influx into growth cones, and CB1 agonist stimulated neurite outgrowth in cerebellar neuron can be blocked by N and L-type calcium channel antagonists (Oudin et al, 2011b; Williams et al, 2003). The calcium ionophore A23187 can also stimulate DAGL and MAGL activity in neutrophils as well as increase 2-AG production in DRG neurons (Balsinde et al, 1991; Gammon et al, 1989). Ionomycin can also significantly increase 2-AG levels in a DAGL dependent manner in COS-7 cells (Bisogno et al, 2003). Calcium influx can also increase 2-AG production in post-synaptic spines (Hashimotodani et al, 2007). However, the mechanism that couples calcium influx to eCB signalling is not known (Oudin et al, 2011b). Our results showed that increased intracellular calcium stimulated by Ionomycin had no effect on the initial rate of DAGL internalisation in COS-7 cells; however it did significantly alter the ratio of cell surface/intracellular DAGLα at 60 min in favour of relatively more being present at the cell surface. Importantly, this could be a consequence of more DAGL transport to the surface, and/or degradation of intracellular DAGL. As mentioned above, translocation of DAGL activity from an intracellular pool towards the plasma membrane was observed by treating the human neutrophils with the calcium ionophore A23187 (Balsinde et al, 1991). However, it is also possible that the Ionomycin response that we observed reflects a

calcium stimulated degradation of DAGLα within the cell, and further experiments will be required to distinguish between these two possibilities. As calcium stimulation can increase 2-AG production, long-term treatment of Ionomycin may have increased 2-AG level to an extent that was sensed by cells, and the cells may respond by reducing the DAGLα level to control the eCB signalling. However, we did see considerable variation between the results obtained in this series of individual experiments (data not shown), and other mechanisms other than calcium influx may also contribute to the DAGLα endocytosis. Further detailed study will be required to confirm the effect of calcium on DAGLα cycling, and to determine the mechanism of any effect.

7.9 The interaction with Homer on DAGLα cellular localization

The Homer protein family consists of three members in mammals (Homer 1-3) (Shiraishi-Yamaguchi & Furuichi, 2007). They are commonly known as scaffold proteins predominantly localized at the postsynaptic density in neurons and are thought to have a role in defining the cellular distribution of the proteins that interact with them (Brakeman et al, 1997; Shiraishi-Yamaguchi & Furuichi, 2007). The interaction between DAGLα and Homer has been reported previously (Jung et al, 2007). This study reported DAGLα and Homer 2 can be co-immunoprecipitated in HEK293 cells. Mutating a single site within a consensus Homer-binding motif PPxxF in DAGLα abolished this interaction. Moreover, one point mutation (P975) in this motif was able to alter the localization of DAGLα from the cell surface to intracellular structures in Neuro-2a cells (Jung et al, 2007). However, our study using a HA-DAGLα-P975L mutant showed that this DAGLα mutant can still be expressed at the cell surface, and this mutation did not obviously impact on the internalization rate of DAGLα, indicating that an interaction with Homer is not required for the transport of DAGLα to the cell surface in COS-7 cells. This conflicting result that we have found might due to the different experimental methods and/or different cell types used in the studies. For example, in Jung et al's study, a C-terminal V5 tagged construct was used and the conclusion was draw from visual inspection of the fluorescent signal, making it more difficult to unequivocally differentiate the surface and intracellular pool of DAGLα. In contrast, in our study we used a surface epitope tagged DAGLα construct and antibody feeding assay, thus the surface expressed DAGLα can be specifically and effectively detected. It is highly possible that the P975L mutation cannot result in a complete change of DAGLα localization from cell surface to intracellular, and the remaining DAGLα on the cell surface was picked up due to the high sensitivity of our assay. It is also possible that the DAGLα-Homer interaction was not

important for DAGLα localization in COS-7 or it is very likely that the dendritic environment or other neuronal specific proteins are required for this interaction. Indeed, by using a Fragile X syndrome model, FMRP was suggested to be involved in correct localization of DAGLα to eCB signalling complexes which includes the scaffolding protein Homer (Jung et al, 2012). Therefore it will be important to further test the effects of the P975L mutation on the localisation of DAGLα to dendritic spines in neuronal cells.

7.10 Conclusions

In summary, by using a novel tagged DAGLα construct that allows us to localise surface expression in live cells, we found a distinct pool of DAGLα expressed on the cell surface that can be differentiated from an intracellular DAGLα pool. Antibody feeding and internalization assays were used to show that DAGLα can be constitutively internalized in several cell lines as well as cultured hippocampal neurons. Detailed co-localization studies showed that DAGLα co-localizes with Homer, a dendritic spine marker, in hippocampal neurons. Studies in COS-7 cells revealed that DAGLα enters the early endosomes after been internalized and can be recycled back to the cell surface possibly via a recycling endosome pathway. Internalized DAGLα does not co-localize with transferrin, also a peptide targeting a putative AP-2 binding site did not show significant effect on DAGLα internalization, suggesting that DAGLα endocytosis might be independent of clathrin and AP-2. DAGLα does not co-localize with Caveolin 1, Flotillin 1 and GPI-linked proteins, markers for some reported clathrin-independent endocytic pathways. These novel findings lead us to postulate that this energy using dynamic recycling of DAGLα is likely to be a tightly regulated process which directly regulates eCB signalling at the synapse as well as other cell types. Understanding the underlying mechanisms in DAGLα endocytosis has therapeutic potential for drug targets in diseases like Fragile X syndrome.

7.11 Future directions

A bioinformatics study scanning the DAGLα sequence might give insights to the underlying mechanisms governing its internalisation. Apart from the putative AP-2 and Homer binding motifs mentioned above, other key motifs or regions have been found that might target DAGLα to the proteasome. In this context, a putative destruction box (D-box) motif has been identified within the C-terminal tail of DAGLα (Gareth Williams, unpublished data). The D-box is the most common sequence recognized by the anaphase-promoting complex (APC), or cyclosome which directs the

ubiquitin-mediated protein degradation (Burton & Solomon, 2001). Therefore the degradation of DAGLα might be controlled by APC-dependent degradation. Indeed, a similar mechanism that regulates the degradation of MAGL, a protein responsible for the major 2-AG degradation, has been reported and the degradation of MAGL has been shown to be regulated by BRCA1, a protein which possesses ubiquitin E3 ligase activity, in cultured cholinergic neurons (Keimpema et al, 2013b).

Evidence has been found to suggest that direct phosphorylation of the catalytic domain within DAGLα is a key regulatory mechanism for the enzyme (Reisenberg et al, 2012) (Dr Praveen K Singh, unpublished data). Phosphorylation may also play a role in DAGLα localization by modulating protein-protein interactions. Indeed, the interaction with CamKII has been reported to be dependent on the phosphorylation of two sites in the tail region of DAGLα (Shonesy et al, 2013). Interestingly, this DAGLα C-terminal tail region is completely absent in DAGLβ (Reisenberg et al, 2012). It will be interesting to test if this tail region is important for endocytosis by generating a C-terminal deleted version of HA-DAGLα or by testing if a chimeric version of DAGLβ containing the tail region from DAGLα localises to, and can be internalised at spines. Constructs to test this are relatively easy to design and indeed some are now available in the Doherty lab. DAGLα and DAGLβ also differ in their short N-terminals which are only15-17 amino acids long. As mentioned above, this N-terminal sequence in DAGLα contains a putative AP-2 binding motif (RRRWS) which is conserved across species but is absent in DAGLβ. Although the peptide 1 targeting this putative AP-2 binding motif didn't show significant effect on DAGLα internalization, it is possible that other sites in this region also play a role. Future studies will be required to identify the regions of the enzyme that are required for internalisation and recycling, and mutagenesis as well peptide mimetic studies are planned. For example, we have designed a construct that swaps the first 15 amino acids of DAGLα to the N-terminal of DAGLβ to test the effect of this on recycling at the cell surface.

The design of tools that specificity inhibit DAGL internalisation, or mutant constructs that cannot be cycled between the intracellular and cell surface compartments, will be required to test the function of dynamic cycling. Our hypothesis is that this will regulate the amount of enzyme on the surface and thereby directly impact on the levels of 2-AG synthesised in response to upstream signals. This in turn should impact on activation of CB1/2 receptors in the same cell (autocrine signalling) or activation of CB1 receptors on the terminals that synapse on DAGLα rich spines

(juxtacrine signalling). The studies reported in the thesis lay the groundwork for the development of these tools and the testing of this exciting novel hypothesis.

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